STRATEGIES TO IMPROVE SYNCHRONIZATION OF OVULATION AND USE OF RECOMBINANT BOVINE SOMATOTROPIN TO ENHANCE EARLY EMBRYONIC DEVELOPMENT AND CONCEPTION RATES IN DAIRY COWS

By

FREDERICO AUGUSTO PENA MOREIRA

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Dedicated to Auricedes, Tereza, e Fernanda.

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

STRATEGIES TO IMPROVE SYNCHRONIZATION OF OVULATION AND USE OF RECOMBINANT BOVINE SOMATOTROPIN TO ENHANCE EARLY EMBRYONIC DEVELOPMENT AND CONCEPTION RATES IN DAIRY COWS

By

FREDERICO AUGUSTO PENA MOREIRA

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A series of experiments was performed to improve the effectiveness of the Ovsynch/TAI protocol and to observe the effects of bovine somatotropin (bST) on pregnancy rates and embryonic development. The Ovsynch/TAI protocol synchronizes time of ovulation and eliminates the need for estrous detection. Lactating dairy cows initiated the Ovsynch/TAI protocol at random stages of the estrous cycle or were pre-synchronized with two $PGF_{2\alpha}$ injections given 14 d apart to increase the percentage of cows initiating the Ovsynch/TAI protocol at the hypothesized optimal stages of the estrous cycle. Pre-synchronization increased first-service pregnancy rates of cyclic but not of anestrous cows. Administration of bST at the day of initiation of

the Ovsynch/TAI protocol or at the time of insemination increased first-service pregnancy rates of cyclic but not of anestrous cows.

Administration of bST at insemination of superovulated cows decreased the percentage of unfertilized ova, increased the percentage of transferable embryos, and enhanced early embryonic. Transfer of bST-treated embryos to recipient cows increased subsequent pregnancy rates. Treatment of recipient cows with bST increased pregnancy rates but there were no additive effects of transferring a bST-treated embryo to a bST-treated recipient.

Addition of bST to maturation medium increased cleavage rates of cultured bovine embryos. Development to blastocyst was increased when bST and insulin-like growth factor-I (IGF-I) were added during embryo culture. Addition of an antibody to IGF-I eliminated the stimulatory effects of IGF-I but not the effects of bST.

In conclusion, pre-synchronization increased pregnancy rates to the Ovsynch/TAI protocol. Administration of bST affected both maternal components and embryonic development to result in increased pregnancy rates.

CHAPTER 1
INTRODUCTION

The reproductive performance of dairy cows has a significant impact on milk production and profitability of dairy operations. Among several measurements of reproductive efficiency, pregnancy rate was considered the most meaningful for cattle producers (Ferguson and Galligan, 1993). Pregnancy rate is the product of estrus detection and conception rates (Macmillan, 1992). Therefore, reproductive performance within a dairy herd is affected greatly by the efficiency of estrous detection and, upon detection of estrus and insemination, by the successful conception and survival of the embryo.

After the advent of artificial insemination, estrous detection became paramount for the success of reproductive management systems. However, detection of estrus is laborious and quite variable depending on season (hot versus cold weather), farm, nutritional and health status, and, ultimately, management skills. Furthermore, intensity of estrus decreases in facilities where cows spend almost all of their time on concrete floors (Hurnik et al., 1975),

making the task of detecting estrus even more difficult in lactating dairy cows confined in free stall facilities.

The development of a timed insemination protocol based on the use of a gonadotropin releasing hormone (GnRH) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) precisely synchronizes the time of ovulation and eliminates the need for estrous detection (Pursley et al., 1995; Burke et al., 1996; Schmitt et al., 1996a). This protocol is designated as the Ovsynch/TAI program. Several experiments tested the Ovsynch/TAI protocol, and, in general, this program produces pregnancy rates greater or comparable to control groups that depend upon estrous detection (Burke et al., 1996; Schmitt et al., 1996a; Pursley et al., 1997a,b; Aréchiga et al., 1998; Britt and Gaska, 1998; De La Sota et al., 1998). However, recent data indicated that stage of the estrous cycle at which the Ovsynch/TAI protocol is initiated influences subsequent pregnancy results (Vasconcelos et al., 1999b; Moreira et al., 2000b). Hence, pregnancy rates may be further increased if estrous cycle stage effects can be circumvented.

Conception rates of dairy cows have been decreasing in the last 40 years (Butler and Smith, 1989). Such an effect is not evident in dairy heifers or in beef cows. A negative association between reproductive efficiency and

milk production has been observed in lactating dairy cows (Butler and Smith, 1989). Therefore, it also is important to study factors that lead to the success or failure of conception after oocyte fertilization and formation of a zygote. Recently, it was observed that the use of bovine somatotropin (bST) was associated with an increase in first-service pregnancy rates when treatment was initiated at the first GnRH injection of the Ovsynch/TAI protocol (Moreira et al., 2000d). Basically, there are two possible mechanisms that may explain this effect: the use of bST at initiation of the Ovsynch/TAI protocol may have increased the efficiency of the synchronization program (e.g., increased ovulation rates or improved quality of the preovulatory follicle). The increase in pregnancy rates would then be associated with bST effects before insemination. Alternatively, administration of bST may have improved pregnancy rates due to its effects postinsemination (e.g., increased fertilization rates, enhanced embryonic development, improved embryonic survival). Thus, it is possible that bST may be enhancing conception rates after insemination and such an effect may counteract the trend for decreasing conception rates observed in the last 40 years. Because bST is widely used among dairies in the United States to increase milk

production, producers also could take advantage of the fact that bST may increase reproductive efficiency of lactating cows. Hence, it is important to verify whether the observed effect of bST is repeatable and to increase our understanding of the mechanisms by which bST increases pregnancy rates.

Objectives of this dissertation were to determine whether pre-synchronization of lactating dairy cows may eliminate problems associated with the stage of the estrous cycle at initiation of the Ovsynch/TAI protocol and increase pregnancy rates after timed insemination. Also, a series of experiments were conducted to examine whether bST increases pregnancy rates of lactating dairy cows after a timed insemination and to further clarify the mechanisms by which bST may influence the reproductive performance of cows. Together, improvement in the degree to which ovulation can be synchronized with the Ovsynch/TAI protocol, coupled with increases in conception rates through administration of bST, have the potential to significantly increase pregnancy rates and to maximize the profitability of dairy operations.

CHAPTER 2
LITERATURE REVIEW

The reproductive performance of dairy cows significantly affects lifetime milk production and profitability of dairy herds. Hence, it is of paramount importance to maximize fertility of dairy herds to optimize economical performance. To achieve such a goal, it is crucial to have a general understanding of the estrous cycle and the many factors that ultimately control follicular development, ovulation, and luteal development to support an eventual pregnancy. It is also important to study factors that may affect embryonic survival. Recent data (Moreira et al., 2000d) indicated that the use of bovine somatotropin may enhance fertility of dairy cows and this effect may be due to the effects of bST on early embryonic development. Therefore, objectives of this literature review are to summarize some aspects related to the physiology of the estrous cycle and embryonic survival; to describe theory and applications of the Ovsynch/TAI protocol; and to review data related to the effects of bovine somatotropin on reproduction.

Estrous Cycle

Estrus in cattle is defined as the period when the female is sexually receptive to be mounted. Duration of this period is described as lasting for 12 to 18 hours in dairy heifers and cows. Recently, Walker et al. (1996) and Dransfield et al. (1998) observed shorter estrous periods (7.4 to 13.6 h and 5.1 to 10.6 h, respectively) using an electronic pressure-sensing system. The estrous cycle is defined as the period between recurring times of sexual receptivity. In cows, the average length is 21 d (Hansel et al., 1973) and this period can be divided into four different stages: proestrus, estrus, metestrus, and diestrus.

During estrus, or the period of sexual receptivity, a dominant preovulatory follicle is present and there is no corpus luteum (CL). This period is followed by metestrus, which lasts approximately 4 d. Ovulation of the dominant follicle occurs during this stage. Luteinization is initiated during estrus and continues, after follicle rupture, with differentiation of the developing CL. The CL is the primary source of progesterone necessary for maintaining pregnancy.

Diestrus is the period of full development and function of the CL. Diestrus starts after differentiation

of the granulosa and theca cells to luteal cells of the CL, and lasts until regression of this structure (i.e., Days 16 to 18 of the estrous cycle). In the presence of a conceptus, the CL will not regress, and the pregnant cow remains in diestrus throughout a pregnancy.

After regression of the CL, the proestrus period is the last stage before estrus or sexual receptivity. During the proestrus period, under a low-progesterone environment after CL regression, the dominant follicle growths rapidly into a fully estrogenic structure that induces estrous behavior. The cycle is completed by estrogenic stimulation of a preovulatory surge of Luteinizing hormone (LH) that induces oocyte maturation and ovulation (Imakawa et al., 1986). The estrous cycle is controlled by endocrine communications among the hypothalamus, pituitary, ovaries, and uterus.

Follicular Development

Upon the development of ultrasonography, it was observed that usually two or three waves of follicular development occur during the estrous cycle (Savio et al., 1988). Each wave of follicular development is divided into three phases: recruitment, selection, and dominance. Follicular development appears to be tightly regulated so

that it usually results in the ovulation of a single dominant follicle at the end of an estrous cycle.

Early Follicular Development

Early follicular growth refers to the development of an ovarian follicle from the primordial to early antral phase (McNatty et al., 1999). The diameter of the ovarian follicle during early development increases from approximately 40 μm (primordial follicle) to 3 mm (early antral). Rajakoski (1960) demonstrated that, within an estrous cycle, 50 to 400 follicles leave the pool of primordial follicles and develop to the early antral stages. However, most of these follicles undergo atresia because only one follicle will ovulate.

Factors that control the initial development of primordial follicles and that lead to atresia of most follicles that leave the primordial pool are not well understood. Early development of primordial follicles seems to be independent from gonadotropins. The FSH receptors are not expressed in primordial follicles. Follicles grow up to 1 to 2 mm in diameter in hypophysectomized sheep (Driancourt et al., 1987) and up to 2 to 4 mm in cattle with diminished FSH and LH secretion (Gong et al., 1996). Furthermore, development of primary

and early antral follicles and a corresponding decrease in primordial follicles were observed when slices of cow ovarian cortex were cultured in gonadotropin-free medium (Braw-Tal and Yossefi, 1997). This observation indicates that initiation of follicular growth is likely to involve paracrine and autocrine rather than endocrine factors (McNatty et al., 1999). Nonetheless, exogenous FSH increased the number of small pre-antral follicles in hypophysectomized mice (Wang and Greenwald, 1993) and the incorporation of [^{3}H] thymidine into pre-antral follicles in hamsters (Roy and Greenwald, 1989). Therefore, although FSH does not seem to be required during early follicular development, evidence suggests that FSH may stimulate granulosa cell proliferation and follicular development at early stages of growth.

Data derived from animal mutant models indicates that several growth factors (e.g., stem cell factor, c-kit, inhibin, activin and follistatin) seem to be involved in the early development process (reviewed by McNatty et al., 1999). In ruminants, expression of these growth factors and their receptors is observed during early follicular growth in a stage- and cell-specific manner (McNatty et al., 1999). These growth factors may thus regulate early follicular development in cattle.

Heat stress also can affect early follicular development. Badinga et al. (1993) and Wolfenson et al. (1995) reported a decrease in Class I follicles (2 to 5 mm) in cows under heat stress. Heat stress reduced the number of small follicles (2 to 5 mm) in heifers (Wilson et al., 1998a) and also tended to reduce them in lactating cows (Wilson et al., 1998b). Although some of these small follicles may be in the recruitment process, Class I follicles usually do not participate in recruitment and probably represent a pool of healthy recruitable follicles as well as follicles undergoing atresia (Wilson et al., 1998b). The decrease in Class I follicles was not associated with an increase in Class II follicles, which indicates that the decline in Class I follicles probably occurred through atresia of earlier stages of follicular development.

Follicular Recruitment

After ovulation or dominant follicle turnover, a group of three to six follicles start to grow larger than 5 mm in diameter. This is defined as the recruitment phase of a follicular wave (Fortune 1994). Evidence indicates that initiation of follicular recruitment is determined by circulating FSH. Follicles do not develop beyond 4 mm in

diameter in cows with diminished FSH concentrations (Gong et al., 1996). Recruitment of follicles is temporally associated with increases in circulating concentrations of FSH (Adams et al., 1992a). Thus, development beyond 4 mm in diameter in cattle is considered to be gonadotropin-dependent.

Other factors may also play a role during the recruitment phase. Bovine somatotropin (bST) and insulin-like growth factor I (IGF-I) receptors are present in ovarian follicles (Izadyar et al., 1997; Kolle et al., 1998; Spicer and Echternkamp, 1994). The number of recruited follicles increased in cows or heifers that were either injected daily with bST or treated with a sustained release formulation of bST (De la Sota et al., 1993; Gong et al., 1991, Gong et al., 1993a; Gong et al., 1997; Kirby et al., 1997a). In contrast, cattle with a deficiency in bST receptors that leads to low IGF-I concentrations had a decreased number of recruited follicles (2 to 9 mm in diameter; Chase et al., 1998). Although it is possible that bST may directly affect follicular recruitment, evidence indicates that IGF-I is probably the main mediator of such an effect (Lucy et al., 2000). Gong et al., (1993a) reported that the increased number of small follicles was sustained throughout the period when

peripheral concentrations of IGF-I remained elevated, even when peripheral concentrations of bST had returned to the levels of untreated cows. One hypothesis to explain the increase in number of follicles recruited in cows treated with bST is that there is a synergistic effect of IGF-I on expression or action of gonadotropin receptors. In vitro studies showed that IGF-I (but not bST) acted synergistically with LH and FSH to stimulate proliferation of cultured bovine granulose cells (Gong et al., 1993b). However, in vivo studies failed to indicate an increase in gonadotropin-binding sites in follicles of cows treated with bST (Andrade et al., 1996). The possibility that IGF-I may increase the activity of gonadotropin second messenger has not been studied. Alternatively, increased IGF-I concentrations due to administration of bST may decrease atresia of growing follicles and results in a greater number of healthy antral follicles. Treatment of cows with bST decreased follicular atresia after estradiol treatment (Cushman et al., 1996). Therefore, decreased atresia of earlier stages of follicular development may partially explain the increase in the number of recruited follicles in cows with high blood IGF-I concentrations. The increase in the number of small follicles caused by bST could also be related to altered function of the dominant

follicle. However, because dominant follicle growth and development are not altered by bST (De la Sota et al., 1993; Gong et al., 1993a), it is unlikely that the increase in the number of small follicles is due to development of a less dominant follicle.

Recruitment may also be affected by nutrition. Increased dietary intake was associated with increased recruitment of small follicles in heifers (Gutierrez et al., 1997). In the latter study, the increased recruitment of follicles was not associated with IGF-I concentrations, which did not differ among treatments. Instead, insulin concentrations were associated with increased development of small follicles. Thus, other hormones besides IGF-I (i.e., insulin) or metabolites (i.e., glucose or fatty acids) may affect follicular growth. Glucose is the primary energy source for the bovine ovary (Rabiee et al., 1999) and abomasal glucose infusion increased the number of recruited follicles in lactating dairy cows (Oldick et al., 1997). Therefore, whole body glucose partitioning should be considered as a mechanism for increased follicular growth after bST treatment (Lucy et al., 2000). Cows fed a diet rich in polyunsaturated fatty acids (i.e., supplemented with soybean oil) had increased follicular development (4 to 9 mm in diameter) compared to cows fed

diets with saturated fatty acids (i.e., animal tallow), highly polyunsaturated fatty acids (i.e., fish meal), or a control diet with no added fat source (Thomas et al., 1997). Such an effect may be due to increased insulin concentrations in cows fed polyunsaturated fatty acids.

It appears that follicles acquire steroidogenic capabilities during the recruitment phase. Initial expression of cytochrome P450 side-chain cleavage and cytochrome P450 aromatase mRNA in granulosa cells occurs when follicles reach 4 to 5 mm in diameter, and it is present in all follicles 6 to 9 mm in diameter (Webb et al., 1999). Induction of these steroidogenic enzymes probably is caused by the transient increase in FSH that precedes each follicular wave (Adams et al., 1992a; Webb et al., 1999). Therefore, it is during follicular recruitment that follicles become able to produce significant amounts of estradiol. Such an observation may have an impact on subsequent FSH concentrations after the recruitment phase, as discussed below.

Follicular Selection

After follicular recruitment, only one follicle is chosen from a cohort of medium-sized growing follicles for further development toward ovulation. Such a process is

defined as follicular selection or follicular deviation. Selection of the dominant follicle in cattle seems to occur between 36 and 48 h after initiation of the follicular wave (Bao et al., 1997a) and when the largest follicle has a mean diameter of 8.5 mm (Ginther et al., 1997, Ginther et al., 1998; Ginther et al., 1999; Kulick et al., 1999). Several growing follicles within a wave have the potential to differentiate into a dominant follicle. This is indicated by the fact that ablation of the dominant follicle 1 or 2 d after the expected beginning of deviation is followed by another follicle in the wave assuming dominance (Adams et al., 1993a). Also, when a 5 mm follicle was chosen at random while other follicles of similar size were ablated, the retained follicle developed the characteristics of a dominant follicle (Gibbons et al., 1997).

Recent data indicated that follicular deviation apparently involves a reduction in systemic FSH concentrations below the concentrations required by the smaller follicles (Ginther et al., 2000). The involvement of low FSH concentrations is based on a temporal relationship between the time of attainment of minimal FSH concentrations and the expected time of deviation (Ginther et al., 1997; Kulick et al., 1999), and an apparent delay

or prevention of deviation after administration of FSH (Adams et al., 1993a; Mihm et al., 1997). Follicles of 5 mm in diameter developed FSH-suppressing capability (Gibbons et al., 1999). The FSH-suppressing capacity at such an early stage seems to be related to estradiol produced by 5 mm follicles (Ginther et al., 2000), but inhibin may be involved in reducing FSH because it is also produced during the selection process (Martin et al, 1991). Although there was no difference in FSH suppression between 5 and 7 mm follicles (Gibbons et al., 1999), a greater FSH-suppression capacity was observed for follicles with an 8.5 mm diameter compared to follicles with a 7.5 mm diameter (Ginther et al., 2000). Such an observation may indicate that the future dominant follicle increases its capacity to suppress FSH as it approaches the time of deviation. During the selection process, the future dominant follicle is producing increasing amounts of both estradiol and inhibin (Martin et al., 1991). The increased production of both estradiol and inhibin may be responsible for increased FSH-suppression as the future dominant follicle grows.

Selection of a dominant follicle appears to be associated with initiation of mRNA expression of the LH receptor (LHR) and 3β-hydroxysteroid dehydrogenase (3β-HSD; Bao et al., 1997a; Xu et al., 1995a). Whether selection of

the dominant follicle or granulosa cell expression of LHR or 3β-HSD occurs first, or whether they occur simultaneously, is unclear. Nonetheless, divergence of the dominant follicle occurs when the circulating concentrations of FSH, which have been decreasing from shortly after recruitment, reaches its nadir (Webb et al., 1999). Hence, it can be hypothesized that the follicle that first acquires LHR in its granulosa cells is able to respond to LH, as well as FSH, and is able to survive in a low FSH environment that is unsuitable to support the development of other follicles (Gong et al., 1996). Because induction of LHR in granulosa cells is dependent on FSH and estradiol in rodents (Segaloff et al., 1990), it can be hypothesized that the follicle with the highest concentration of estradiol during recruitment would be the first follicle to develop LHR in the granulosa cells and, therefore, be able to survive declining concentrations of FSH. In support of this hypothesis, experiments have indicated that emergence of the future dominant follicle (as a 3 to 4 mm diameter follicle) occurred at a mean of 6 to 7 h before the emergence of the future largest subordinate follicle, and the dominant follicle maintained a diameter advantage of approximately 0.5 mm in diameter until deviation (Ginther et al., 1997, Kulick et al.,

1999). Therefore, a follicle that has an initial advantage in size as compared to other follicles in early stages of follicular development may be the follicle that will produce more estradiol and first develop LHR.

Follicular Dominance

The dominance period within a follicular wave is characterized by the presence of a large (>10 mm in diameter) follicle that suppresses the development of smaller follicles. Sirois and Fortune (1990) defined the dominant follicle as a follicular structure whose diameter is 2 mm greater than other follicles. Furthermore, the dominant follicle was classified as "estrogen active" based on a positive ratio of estradiol relative to progesterone concentrations in the follicular fluid (Ireland and Roche, 1983).

The dominance phenomenon is not understood completely. However, it is clear that the dominant follicle secretes compounds that inhibit follicular development. Because follicular development is inhibited both in the ovary that bears the dominant follicle and in the contralateral ovary, this inhibition is probably at the endocrine level. Infusion of proteinaceous fractions of follicular fluid into cyclic heifers prevented the process of recruitment

from occurring during the first 5 d of the estrous cycle (Kastelic et al., 1990). Injection of follicular fluid in heifers also reduced circulating FSH concentrations (Lussier et al., 1994). Ireland et al. (1994) hypothesized that compounds present in follicular fluid such as inhibin may decrease the circulating concentrations of FSH and avoid further follicular growth. Inhibin is a gonadal peptide that has increased circulating concentrations during the period of dominance and has been shown to selectively suppress secretion of FSH (Padmanabhan et al., 1984). Furthermore, dominant follicles acquire an increased capability to produce steroids during their development (Xu et al., 1995b; Bao et al., 1997a), and estradiol induced a decrease in FSH (Ginther et al., 2000).

As discussed above, FSH concentrations decrease during the selection process and remain low throughout the dominance period. The dominant follicle appears to be able to survive in a low FSH environment by switching some of its gonadotropin requirements from FSH to LH (Bao and Garverick, 1998. However, recent experiments indicate that the low concentrations of FSH are still required for continued growth of the dominant follicle (Ginther et al., 2000). Furthermore, dominant follicles express greater levels of steroidogenic acute regulator protein (StAR) in

theca cells (Bao et al., 1997b), and this may assure sufficient cholesterol transport across the mitochondria for androgen production. In addition, granulosa cells of dominant follicles express high levels of mRNA for aromatase, which may enable them to convert large amounts of androgens produced by the theca cells into estradiol (Xu et al., 1995b; Bao et al., 1997b). Estradiol can enhance granulosa cell responsiveness to FSH in rats (Richards et al., 1980) and, therefore, increased estradiol concentrations in follicular fluid of dominant follicles have the potential to increase sensitivity to FSH in cattle (Henderson et al., 1984).

The increased steroidogenic capacity of dominant follicles may be of paramount importance for its persistence. Such an increased steroidogenic capacity is associated with a pulsatile secretion of LH (Webb et al., 1999). Exogenous pulses of LH cause persistence of dominant follicles in cows (Taft et al., 1996). Insertion of a progesterone-releasing device after luteolysis also caused an increased persistence of the dominant follicle (Savio et al., 1993b). However, if the LH pulse frequency is too low, as occurs during diestrus, further growth of the dominant follicle is impaired. Interestingly, IGF-I may sensitize the dominant follicle to the effects of LH

and increase its steroidogenic capacity. It was observed that IGF-I is a potent stimulator of granulosa cell function in the bovine, including both proliferative and steroidogenic activity (Gong et al., 1991; Spicer et al., 1993). In bovine theca cells, IGF-I increases the number of LH-binding sites and enhances LH-induced production of progesterone and androstenedione in vitro (Stewart et al., 1996), thereby increasing the production of substrate for subsequent conversion to estradiol in granulosa cells.

Because LH pulsatility affects the production of estrogens, the dominant follicle becomes atretic if luteolysis does not occur during its growing phase (Webb et al., 1999). Expression of gonadotropin receptor mRNAs, steroidogenic enzymes, and StAR decrease rapidly with atresia, and a decline in expression occurs earlier than morphological signs of atresia are observed (Xu et al., 1995b). Atresia of dominant follicles appears to be initiated between Days 4 and 6 of dominance in the first follicular wave (Webb et al., 1999). Similarly, expression of mRNAs for FSHR and P450 side chain cleavage enzyme in granulosa cells, and LHR in theca cells decrease markedly between Days 4 and 6 (Webb et al. 1999).

Other factors such as bST also affect the dominance period. Although the size of the dominant follicle was not

affected by bST treatment (De la Sota et al., 1993; Kirby et al., 1997a), the duration of the dominance phase was shortened by about 2 d in bST-treated cows (Kirby et al., 1997a; Lucy et al., 1994b). Therefore, greater concentrations of bST and IGF-I may accelerate the series of events that lead to follicular atresia and cause premature loss of follicular dominance (Lucy, 2000). This phenomenon of reduced dominance in bST-treated cows was associated with an increased expression of low molecular weight binding proteins in the follicular fluid (Lucy et al., 1995b; Thatcher et al., 1996). Atretic follicles have greater amounts of IGF-I binding proteins (IGFBPs; De la Sota et al., 1996). Therefore, the increase in IGFBPs in cattle treated with bST may cause premature atresia and loss of dominance.

Heat stress also can affect the dominant follicle. Badinga et al. (1993) and Wolfenson et al. (1995) demonstrated that follicular dominance was impaired in lactating cows during periods of heat stress. The dominant follicle of the first follicular wave was found to be smaller in diameter and to contain less follicular fluid in heat stressed cows (Badinga et al., 1993). Similar observations were made when both lactating cows and heifers were submitted to heat stress (Wilson et al., 1998). Also,

the latter report observed that the decreased follicular size in heat stressed cattle was associated with decreased steroidogenesis. Possible mediators of the heat stress response are reduced viability of granulosa cells or changes in steroidogenic enzymes (Wilson et al., 1998). In vitro, heat stress reduced androstenedione and estradiol production from follicular cells of dominant follicles (Wolfenson et al., 1997). A similar mechanism may be present in vivo.

Co-dominance and Multiple Ovulations

Co-dominance may be defined as the development of two or more dominant follicles with a follicular wave. Development of co-dominance may result in multiple ovulations in cattle. In general, co-dominance may result after failure of the mechanisms of follicular selection or after pharmacological treatment of cattle or.

Several superovulatory treatments for cattle based upon the use of FSH or PMSG have been described in the literature (Gong et al., 1993, Gong et al., 1996). These superovulatory treatments are based on the use of FSH or PMSG (i.e., FSH-like activity) to support follicular growth. Thus, superovulation treatments are able to bypass

the inhibition of FSH by the dominant follicle and result in the development of multiple dominant follicles.

Multiple ovulations may result from genetic factors. Although the heritability for twinning rate is low, progress is possible when cattle are selected for twinning (Echternkamp et al., 1990). Because most twins in dairy cattle are dyzygous (Ryan and Boland, 1991), twinning rates may be associated with increased incidence of co-dominance. The reason why the selection of a single follicle is inhibited in such a group of cows is unknown. Among several possible alternatives, either the mechanisms by which the largest follicle exerts its dominance (i.e., reduction of FSH by estradiol or inhibin) are impaired or the pituitary is not as responsive to the negative feedback of estradiol or inhibin on the production of FSH.

It also has been proposed that the use of bST induced the occurrence of co-dominance and resulted in multiple ovulations (Cole et al., 1992). The increase in twinning rates, however, has not been duplicated in subsequent studies (Collier et al., 1997). Although a direct effect of bST on the selection of dominant follicles is not evident, indirect effects of bST may influence the selection process. Use of bST causes a transient negative energy balance in lactating cows (Etherton and Bauman,

1998) and may therefore indirectly lead to a decreased LH pulsatility (Canfield and Butler, 1990). Such a reduced LH pulsatility could result in shorter dominance phases and greater growth of subordinate follicles (Lucy, 2000). Therefore, energy balance may negatively affect the dominant follicle and result in co-dominance. Reduced energy intake in postpartum beef cows reduced the size of dominant follicles and the number of large estrogen-active follicles and increased the persistence of subordinate follicles (Perry et al., 1991). Negative energy balance in postpartum dairy cows was associated with an increased number of medium-sized follicles and decreased maximum diameter of dominant follicles, whereas positive energy balance was associated with increased maximum diameter of dominant follicles and reduced growth of subordinate follicles (Lucy et al., 1991). If the dominance mechanism by which the growth of smaller follicles is inhibited is disrupted or delayed by lower LH pulsatility, there is a chance that the subordinate follicles may also acquire the capacity to respond to LH (i.e., acquire LHR) and also survive under low FSH concentrations. Thus, co-dominance would result.

In a similar manner, heat stress may also result in co-dominance. Because heat stress seems to result in a

less dominant follicle (Badinga et al., 1993; Wolfenson et al., 1995), subordinate follicles may escape the dominance effect and develop receptors for LH. If the FSH-suppression by the dominant follicle is inhibited by negative energy balance or heat stress, subordinate follicles also may survive long enough to acquire LHR. Estradiol production of dominant follicles is reduced (Wolfenson et al., 1997) and aromatase activity was diminished (Badinga et al., 1993) under heat stress conditions. Moreover, the number of Class III follicles (> 9 mm in diameter) is increased under heat stress (Wolfenson et al., 1995).

Recently, it was observed that parity and milk production was associated positively with the incidence of double ovulations in dairy cows (Fricke and Wiltbank, 1999). Previously, milk production also was associated positively with increased twinning rates (Kinsel et al., 1998). It has been proposed that increased dry matter consumption (which is observed among cows with high milk production) may cause greater clearance of progesterone by the liver (Vasconcelos et al., 1998). Furthermore, the rate of double ovulations was increased in cows with lower progesterone concentrations during follicular development (Vasconcelos et al., 1999a). Collectively, these

observations suggest that the incidence of co-dominance is increased when follicular growth occurs under lower plasma progesterone concentrations. The reasons for such an effect are unknown. These changes in steroid metabolism may alter the endocrine environment sufficiently to allow for selection and deviation of two follicles during the selection process. However, such a hypothesis seems to contradict the expected effects of negative energy balance and heat stress discussed above because, at this time, lower plasma progesterone would probably result in an increased LH pulsatility.

Ovulation, Formation and Regression of the Corpus Luteum

LH Secretion During the Periovulatory Period

The preovulatory surge of gonadotropic hormones from the anterior pituitary gland is the pivotal regulatory step in the estrous cycle because it induces ovulation (Caraty et al., 1995). Hence, great efforts have been made to gain an understanding of the factors that regulate the occurrence of the preovulatory surge of LH and FSH.

It has been demonstrated that increasing concentrations of estradiol in plasma after the decline in plasma progesterone will induce a preovulatory surge of LH in cows (Kesner et al., 1981). Hence, estradiol could

initiate the preovulatory surge of LH by acting on the hypothalamus to increase secretion of GnRH or at the pituitary level to increase the sensitivity of the gonadotrophes to GnRH. In ovariectomized ewes, treatment with estradiol induces a biphasic pattern of LH release in which there initially is an inhibition in LH secretion followed by a large release of LH similar to the spontaneous preovulatory LH surge (Pelletier and Signoret, 1969). It was further demonstrated that the estradiol-induced biphasic release of LH also produced a biphasic pattern in the secretion of GnRH coincident with LH secretion (Caraty et al., 1995). It also was concluded in this study that a massive and sustained release of GnRH is necessary for the occurrence of the LH surge, and estradiol seems to be acting on the ventromedial nucleus of the hypothalamus to cause a switch in the mode of GnRH secretion from strictly episodic to a sustained increase of GnRH in portal blood.

The preovulatory surge of LH lasts for approximately 10 hours (Chenault et al., 1975; Chenault et al., 1976; Thatcher and Chenault, 1976; Rahe et al., 1980; Cupp et al., 1995). Before the pre-ovulatory surge of LH, concentrations of this hormone fluctuate with a high frequency/low amplitude pattern that is coupled with a

decrease in progesterone and an increase in estradiol in the circulation (Rahe et al., 1980; Walters et al., 1984; Walters and Schallenberger, 1984; Cupp et al., 1995). This increased frequency in pulsatile secretion of LH may have important effects on the final development of the preovulatory follicle. McNatty (1979) proposed that growth and differentiation of the ovulatory follicle during the preovulatory stage dictates the formation of a functional CL. In primates, inappropriate patterns of pituitary gonadotropins in the circulation lead to abnormalities in the developing dominant follicle, which resulted in a deficiency in estrogen secretion and an inability of granulosa cells to luteinize appropriately (DiZerega and Hodgen, 1981). Furthermore, Greeve et al. (1995) reported that a reduction in concentrations of circulating LH during the period from luteolysis to the preovulatory LH surge altered maturation of the oocyte.

No further release of LH was detected for the next 12 hours after the LH surge was detected in cattle (Walters and Schallenberger, 1984). However, secretion of GnRH seems to last for hours after the end of the LH surge in sheep (Caraty et al., 1995). This uncoupling is probably due to a desensitization of the pituitary to GnRH. Down-regulation of pituitary receptors for GnRH was induced

after infusion of GnRH for 16 hours (Nett et al., 1981). This was further confirmed in cattle by a decrease in GnRH receptors in the pituitary of cycling heifers after estrus (Schoenemann et al., 1985). In cattle, consecutive injections of GnRH every 4 hours for 96 hours induced a release of LH after each injection, but the amplitude and duration of the release of LH was decreased considerably after the first injection (Kinder et al., 1975). From day 2 to day 4 after detection of behavioral estrus, a pulsatile high frequency and low amplitude pattern of LH release was reported (Rahe et al., 1980; Peters et al., 1994; Cupp et al., 1995). This period of high LH pulsatility is essential for the development and maintenance of the CL (Burke et al., 1994; Peters et al., 1994).

Differentiation of Granulosa and Theca Cells into Luteal Cells

Luteal function and progesterone secretion are essential for regulation of the estrous cycle as well as establishment of pregnancy (Hansel and Convey, 1983; Wilmut et al., 1986; Thatcher et al., 1994). The CL consists of a heterogeneous population of cells that have distinct morphological, endocrinological, and biochemical properties (Niswender and Nett, 1988). Cell types identified in the

CL include small luteal cells, large luteal cells, fibroblasts, endothelial cells, and pericytes (Hansel et al., 1991). In cattle, steroidogenic cells are the small and large luteal cells with basal progesterone secretion greater for large luteal cells compared to small luteal cells (Urseley and Leymarie, 1979). Niswender et al. (1985) calculated that 80% of the progesterone secreted in vivo by the CL of sheep is secreted by large luteal cells. The follicular lineage of steroidogenic luteal cells has been a topic of investigation for many years. In sheep and cattle, the general consensus is that granulosa cells differentiate into large luteal cells and small luteal cells originate from theca cells (O'Shea, 1987). In ewes, the number of granulosa and theca cells in the preovulatory follicle approximate the number of large and small luteal cells respectively in the CL (O'Shea et al., 1980; Rodgers et al., 1984). Furthermore, bovine granulosa and theca cells were cultured and differentiated into large and small luteal cells respectively (Meidan et al., 1990). However, studies have suggested that small luteal cells also differentiate into large luteal cells and that there may be a population of stem cells in the CL, which differentiate into small luteal cells, which in turn differentiate into large luteal cells (Niswender et al., 1985). Niswender et

al. (1985) also reported that transformation of small luteal cells into large luteal cells could be increased by treatment with hCG in vivo. In later studies, treatment of ewes with pharmacological concentrations of LH induced the formation of cells intermediate between small and large luteal cells (Farin et al., 1988). When ewes were treated with physiological concentrations of LH, these intermediate cells were not observed. Injection of hCG at day 5 of the estrous cycle in cows induced an accessory CL that was larger and more steroidogenic than an accessory CL induced by an injection of GnRH (Schmitt et al., 1996b). Authors hypothesized that the greater LH-like activity of hCG may have increased the differentiation of fibroblasts into small luteal cells and differentiation of small luteal cells into large luteal cells. Those observations suggest that small luteal cells may respond to LH or LH-like stimulation and differentiate into large luteal cells. Also, the magnitude of LH-stimulated progesterone secretion is greater in small luteal cells compared to large luteal cells (Hansel et al., 1991).

Luteolysis

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the primary luteolytic agent in farm animals. Secretion of $PGF_{2\alpha}$ from the uterus

in a series of 5 to 8 discrete pulses at 6 to 8 hour intervals induces regression of the CL in cattle (Silvia et al., 1991). A period of progesterone priming is required for the synthesis of $PGF_{2\alpha}$ by the endometrium (Geisert et al., 1992). Progesterone increases phospholipid stores, which increases the potential pool of arachidonic acid, and also increases the activity of the cyclooxygenase enzyme, which converts arachidonic acid to $PGF_{2\alpha}$ (Eggleston et al., 1990). In ewes, progesterone also inhibits the synthesis of oxytocin receptors by the endometrium during the first 10 to 12 d of the estrous cycle (McCracken et al., 1984). After that period, progesterone down-regulates its own receptor, which allows for a rapid increase in endometrial oxytocin receptors that is induced by estradiol (McCracken et al., 1984; Knickerbocker et al., 1988). The loss in progesterone inhibition is thought to be an initiating factor in luteolysis as it allows oxytocin receptor formation. However a more complex mechanism seems to be in place (Wathes and Lamming, 1995).

Silvia et al. (1991) proposed a model in which oxytocin released from the posterior pituitary gland is secreted in a pulsatile fashion causing a uterine release of $PGF_{2\alpha}$. The low concentration of $PGF_{2\alpha}$ released in

response to neurohypothyseal oxytocin triggers the release of additional oxytocin from the CL. The additional oxytocin secreted by the CL stimulates a greater release of $PGF_{2\alpha}$ from the uterus that results in CL regression. However, there is still controversy as to the identity of the initial stimulus for $PGF_{2\alpha}$ secretion. Kotwica et al. (1997) treated heifers with an antagonist to oxytocin (which acts as a competitor for oxytocin receptors) and such a treatment did not affect duration of the estrous cycle or concentrations of either progesterone or $PGF_{2\alpha}$ metabolite. Thus, if ovarian oxytocin and uterine/ovarian oxytocin receptors are involved in luteolysis in cattle, they play a more facilitatory than mandatory role.

Receptors for $PGF_{2\alpha}$ are located on large luteal cells (Chegini et al., 1991). It has been reported that $PGF_{2\alpha}$ has antisteroidogenic effects mediated through a protein kinase C (PKC) second messenger system and induced intracellular release of calcium (Niswender et al., 1994). The release of free calcium may induce apoptosis in large luteal cells (Juengel et al., 1993). The reason for regression of small luteal cells is not known, but secretion of a cytotoxic factor from large luteal cells and the involvement of immune cells have been suggested (Pate, 1994). In

addition, $PGF_{2\alpha}$ increases mRNA for stress-related or apoptotic genes (McPherson et al., 1993; Rueda et al., 1995; Rueda et al., 1997) and also increases mRNA for factors involved in immune responses (Pate, 1994; Townson et al., 1996; Tsai et al., 1997). In addition to regulation of gene expression, $PGF_{2\alpha}$ also causes acute morphological and physiological changes in luteal cells such as decreased membrane fluidity (Carlson et al., 1982), depletion of antioxidants from the CL (Sato et al., 1974), and increased activity of phospholipases and proteolytic enzymes (Sawada and Carlson, 1991). More recently, it also was observed that $PGF_{2\alpha}$ increased mRNA encoding enzymes that stimulate $PGF_{2\alpha}$ production in the ovine CL (Tsai and Wiltbank, 1997). The combination of these $PGF_{2\alpha}$-induced changes results in cessation of luteal progesterone production and involution of luteal tissue (Tsai and Wiltbank, 1998).

Soon after $PGF_{2\alpha}$ was discovered to be the primary luteolytic factor in ruminants, it was observed that injection of $PGF_{2\alpha}$ fails to induce CL regression during the first days of the estrous cycle (Lauderdale, 1972; Hafs et al., 1974). The reason for such a failure is not understood completely. High-affinity receptors for $PGF_{2\alpha}$

are present in the bovine CL as early as day 2 of the estrous cycle (Wiltbank et al., 1995). Furthermore, there were no differences in either affinity or concentration of receptors for $PGF_{2\alpha}$ between early (Days 2 and 4 of the estrous cycle) and active (Days 6 and 10 of the estrous cycle) CL in dairy heifers (Wiltbank et al., 1995). Hence, lack of responsiveness of the early CL is not due to lack of high-affinity receptors for $PGF_{2\alpha}$. Tsai and Wiltbank (1998) reported differential effects of $PGF_{2\alpha}$ in early and mid-cycle CL. The mid-cycle CL has the capacity to respond to $PGF_{2\alpha}$ and induce the production of intraluteal $PGF_{2\alpha}$ (via induction of the PGHS-2 enzyme). Also, mid-luteal CL responded to $PGF_{2\alpha}$ treatment with an increase in the expression of monocyte chemoattractant protein-1 (MCP-1), which is associated with increased luteal accumulation of immune cells. These same responses were diminished in the early CL and thus may help explain the inability of the early CL to undergo regression after $PGF_{2\alpha}$ treatment. Hence, such data suggest that the inability of the early CL to respond to $PGF_{2\alpha}$ may be due to uncoupling of the $PGF_{2\alpha}$ receptor and its second messenger system. Recently, it was demonstrated that administration of $PGF_{2\alpha}$ at d 4 of the estrous cycle does not induce the expression of endothelin-

1 or its receptor by endothelial cells lining blood vessels of the developing CL (Levy et al., 2000). Endothelin-1 had been identified previously as a mediator of $PGF_{2\alpha}$-induced luteal regression (Meidan et al., 1999). These results may indicate that other cell types such as the endothelium may also be unresponsive to $PGF_{2\alpha}$ during the early luteal phase.

Howard and Britt (1990) conducted an experiment in which heifers were injected with hCG at day 10 of the estrous cycle to induce an accessory CL. After injection of hCG (experimental d 0), heifers were injected with $PGF_{2\alpha}$ at 2, 4, and 6 d later to induce luteolysis. Results indicated that luteolysis of endogenous and accessory CL had occurred not only in heifers injected with $PGF_{2\alpha}$ at 6 d after hCG as expected, but also in all heifers injected with $PGF_{2\alpha}$ at 2 and 4 d after induction of an accessory CL. The reason why the newly formed CL underwent complete regression after injection of $PGF_{2\alpha}$ was unclear. Nonetheless, authors hypothesized that an accessory CL induced in an environment of elevated progesterone concentrations is able to respond to $PGF_{2\alpha}$ and regress at earlier stages of development.

Postpartum Anestrus in Cattle

Anestrus denotes a state of complete sexual inactivity with no manifestation of estrus. In cattle, a period of anestrus follows parturition and continues during lactation. The initial block to cyclicity appears to be due to the inhibitory effects of high steroid concentrations secreted during pregnancy at the hypothalamus and pituitary level (Peters and Lamming, 1990). However, in cattle, concentrations of estradiol in peripheral blood decrease sharply at parturition to basal concentrations (1 to 8 pg/mL) within 2 to 6 d (Echternkamp and Hansel, 1973; Humphrey et al., 1983). A rather synchronous emergence of new follicular waves 5 to 7 d postpartum in multiparous dairy cows was observed (Beam and Butler, 1997). Formation of follicular waves after parturition begins synchronously in response to increased FSH in the first week postpartum, and is typically not a limiting factor in reproductive recrudescence (Beam and Butler, 1999). Recent data has demonstrated that postpartum dairy cows can respond to GnRH agonist implant and ovulate as early as 7 d postpartum (Mattos et al., 2000). Thus, the period necessary for the recovery of secretory capacity of the gonadotrophes in the pituitary

seems to be short. Presently, the average day to first ovulation is approximately 25 to 30 d postpartum with a typical range between 17 and 42 d (Butler and Smith, 1989). Collectively, data indicates that the reason for such a delayed ovarian recrudescence is not a prolonged refractory period of the pituitary, but perhaps a period of absence of GnRH stimulus by the hypothalamus.

The duration of the anestrous period varies greatly depending on several different factors such as breed, parity, nutritional status, level of milk production, body condition status at parturition, suckling, problem calving, and the occurrence of puerperal disturbances. Among these factors, suckling and nutrition (i.e., negative energy balance postpartum) seem to play major roles in the postpartum interval to first ovulation.

In beef cows, suckling is considered to be a main factor to influence the length of the anestrus period (Short et al., 1990). The risk of beef cows to be in anestrus at 70 d postpartum was 8.1 times greater for suckled cows (Ducrot et al., 1994). Suckling inhibits the release of LH and delays first ovulation in cattle for an average of 45 to 60 d (Gazal et al., 1998). This suggests that nursing or frequency of milk removal influences the pituitary gonadotropic activity. However, the duration of

anestrus in cows nursing calves is longer than in similar cows milked twice daily. Furthermore, regular milking of dairy cows is less inhibitory to spontaneous LH release than the combination of milking and nursing (Carruthers et al., 1980). Viker et al. (1989) using mastectomized cows indicated that just the presence of a calf causes the same inhibition in postpartum ovulation as in an intact cow that is suckled by her calf. Zalesky et al. (1990) demonstrated that the inhibitory effects of suckling are expressed at the hypothalamus and not on the pituitary. Inhibition of LH release may be caused by high cortisol concentrations occurring during suckling or milk removal (Wagner and Li, 1982).

Also, an opioid-mediated mechanism may be responsible for the suckling-induced LH suppression in cows (Whisnant et al., 1986). Malven et al (1986) suggested that suckling suppresses estrous cycles by influencing endogenous opioid concentrations, which, in turn, affect the release and/or synthesis of hypothalamic GnRH. Administration of naloxone, an opioid antagonist, 24 to 35 d after parturition increased average concentrations of LH in cattle (Gregg et al., 1986). However, naloxone treatment postpartum did not elicit improvements in reproductive performance of beef cows (Jaeger et al., 1995).

Furthermore, suckling is believed to increase sensitivity of the hypothalamus to the initial negative feedback of ovarian estradiol (Acosta et al., 1983; Garcia-Winder et al., 1984), which results in delayed resumption of LH pulsatility (Lamming et al., 1982) and anovulation.

The period of negative energy balance (NEB) typical of early lactation in high-producing dairy cattle has been associated with delayed reproductive recovery, and energy balance after parturition is correlated negatively with days to first ovulation (Butler et al., 1981; Ducker et al., 1985). Similarly, nutrient intake also was demonstrated to influence the interval from calving to first ovulation in beef cows (Spitzer et al., 1995). In a recent epidemiological study (Opsomer et al., 2000), change in body condition within 30 or 60 d after calving was the most influential risk factor that resulted in delayed ovulation among dairy cows. Beef cows loosing one unit or more than one unit in body condition score within 2 months of lactation were 2.6 times at a greater risk to be anestrus at 70 d postpartum (Ducrot et al., 1994). Anestrus can be induced in cyclic beef cows by nutrient restriction (Wettemann and Bossis, 1999). Anovulation and cessation of estrous cycles occurred in beef cows and heifers that lost 22 to 36% of their body condition (Bossis

et al., 1999; Richards et al., 1989; Rhodes et al., 1995). Realimentation of nutritionally induced anovulatory cows resulted in resumption of estrous cycles, but cows did not reinitiate estrous cycles until body condition score was greater than at which cycles ceased (Richards et al., 1989).

Follicular development was affected by energy balance in several different reports. Reduced energy intake in postpartum beef cows reduced the size of dominant follicles and the number of large estrogen-active follicles, and increased the persistence of subordinate follicles (Perry et al., 1991). NEB in postpartum dairy cows was associated with an increased number of medium-sized follicles and decreased maximum diameter of dominant follicles, whereas positive energy balance was associated with increased maximum diameter of dominant follicles and reduced number of subordinate follicles (Lucy et al., 1991). Such an effect on follicular development is probably associated with secretion of gonadotropins.

Frequent LH pulses are necessary for the development and maturation of dominant follicles (Robertson et al., 1989; Stock and Fortune, 1993). Suppression of pulsatile LH secretion in cattle with a GnRH agonist resulted in a substantial decrease in the maximum size of the dominant

follicle (Gong et al., 1995). Pulsatile secretion of LH is inhibited when feed intake is severely reduced in beef cows (Day et al., 1986; Richards et al., 1989). An increase in LH pulse frequency followed the energy balance nadir in lactating cows (Canfield and Butler, 1990). Furthermore, a reduction in size of dominant follicles was reported in feed-restricted beef cows compared with adequately fed cows, and this was associated with reduced LH pulse frequency (Perry et al., 1991). Thus, there is evidence that LH secretion is affected by energy balance and is in part responsible for alterations in follicular development during anestrus. In contrast, similar secretion of FSH in restricted-fed and maintenance-fed heifers before the onset of nutritionally induced anovulation and increased concentrations of FSH in serum after the onset of anovulation indicate that the secretion of FSH is not the limiting factor in nutritionally restricted cattle (Wettemann and Bossis, 1999).

Alterations in LH secretion patterns during NEB suggest that secretion of GnRH from the hypothalamus also is affected. Pituitary responsiveness to GnRH is not compromised during restriction of feed intake in cattle (Beal et al., 1978; Wishnant et al., 1985; Rasby et al., 1992). Thus, anestrus suppression of LH pulses is probably

an effect of altered hypothalamic function. Interestingly, though, concentrations of GnRH in the stalk median eminence of nutritionally anestrous beef cows are greater than in fatter cows that exhibit normal estrous cycles. Therefore, it is possible that, although anestrus increases concentrations of GnRH, the pulse generator within the hypothalamus is affected and results in the inhibition of LH pulses. Indeed, pulsatile infusion of GnRH every hour initiated luteal activity in nutritionally anovulatory beef cows (Bishop and Wettemann, 1993; Vizcarra et al., 1997). Because the effects of nutrition on anestrus appear to be exerted at the level of the hypothalamus, several investigators are actively searching for the metabolic signal that re-initiates normal hypothalamic function. At this point, the two main candidates are leptin and insulin-like growth factor I (IGF-I).

Leptin is a 16-kDa-protein hormone secreted from white adipocytes that has been implicated in the regulation of food intake. Secretion of leptin is decreased during periods of food deprivation and increased with feeding and body fat content (Hamann and Matthaei, 1996; Houseknecht et al., 1998). Based on data from rodents and humans, plasma leptin is expected to decrease after parturition when dairy cows mobilize adipose tissue to support lactation

(Ingvartsen and Andersen, 2000) and to increase after increased dry matter consumption and alleviation of the NEB. Thus, leptin could provide an accurate, circulating signal of nutritional status and be the long-sought indicator that allows reproductive process to proceed (Houseknecht et al., 1998). It has been hypothesized that leptin could act at the level of hypothalamus through its effects on the secretion of neuropeptide Y.

Neuropeptide Y (NPY) is found in cell bodies and nerve terminals in many areas of the brain, particularly those involved in intake regulation and energy balance (Ingvartsen and Andersen, 2000). When administered in the central nervous system, NPY is one of the most potent inducers of food intake (Stanley et al., 1986). This peptide also is expressed highly in the hypothalamus, anterior pituitary, and adipose tissue, and negatively modulates the secretion of LH when centrally infused (Carro et al., 1997; Dyer et al., 1997; McDonald et al., 1989). Gazal et al. (1998) demonstrated that the complete suppression of LH pulses occurred coincidently with a corresponding inhibition of GnRH pulses. The increase of NPY production has been postulated to decrease the stimulatory input to downstream neural pathways that ultimately reach GnRH neurons (Campfield et al., 1996;

Schwartz et al., 1996). Thus, there is reason to believe that the effects of NEB on hypothalamic GnRH release could be mediated by NPY. Leptin receptors have been localized on hypothalamic NPY neurons in sheep (Houseknnecht et al., 1998). Leptin administration decreases NPY expression in the arcuate nucleus (Campfield et al., 1996; Schwartz et al., 1996), presumably removing the inhibition in GnRH release. Thus, there is growing evidence that leptin may constitute an important factor leading to resumption of reproductive function in anestrus cows. Further research is warranted to increase our understanding of the role of leptin in nutrition-reproductive interactions.

There is evidence that IGF-I also may play an important part in the resumption of reproductive function in cattle. In beef cattle, circulating levels of IGF-I were associated with the capacity of anestrous cows to resume a cyclic status (Burns et al., 1997; Roberts et al., 1997). Similar observations were made in dairy cows (Beam and Butler, 1997). Circulating concentrations of IGF-I decline at parturition and gradually increase over time, whereas concentrations of somatotropin (ST) increase at parturition and then decline over time (Ronge et al., 1988; Schams et al., 1991; Vicini et al., 1991). The magnitude of decline and duration of time required for IGF-I

concentrations to return to prepartum levels are greater in animals subjected to dietary restriction (Ronge et al., 1988; Spicer et al., 1990; Ryan et al., 1994), and concentrations of IGF-I were correlated positively with energy balance (Beam and Butler, 1998; Ronge et al., 1988; Spicer et al., 1990). Under adequate dietary availability, circulating concentrations of IGF-I are under positive regulation by ST (Cohick et al., 1989). However, dietary restriction results in elevated circulating ST, suppressed IGF-I, and a loss of IGF-I responsiveness to bST treatment (McGuire et al., 1992). The loss of IGF-I responsiveness to bST in dietary-restricted cattle may be due to decreased hepatic binding sites for GH (Breier et al., 1988).

The mechanism of action of IGF-I, however, differs from the model proposed for the actions of leptin. A review of studies comparing lactating and nonlactating cows indicated that dominant follicles of lactating dairy cows in NEB have a lower estradiol output per unit of gross follicular size compared to nonlactating cows in positive energy balance (Beam and Butler, 1999). Furthermore, rising concentrations of estradiol during the postpartum period are believed to stimulate LH secretion by the pituitary. Differences in metabolic hormones, such as IGF-I and insulin, could be involved in follicular development.

It was observed that IGF-I is a potent stimulator of granulosa cell function in the bovine, including both proliferative and steroidogenic activity (Gong et al., 1991; Spicer et al., 1993). In bovine theca cells, IGF-I increases the number of LH-binding sites and enhances LH-induced production of progesterone and androstenedione in vitro (Stewart et al., 1995). Concentrations of serum IGF-I were positively correlated with plasma concentrations of estradiol, and it was hypothesized that decreased IGF-I might have contributed to reduced steroidogenesis, thereby preventing final follicle maturation and the production of LH surge-inducing concentrations of estradiol (Beam and Butler, 1998).

Previous experiments have demonstrated that treatment of lactating dairy cows with bST increased preovulatory follicular growth, estrogen secretion and size of the subordinate follicle (De la Sota et al., 1993; Lucy et al., 1993). Moreover, cattle with a genetic deficiency in ST receptors have low peripheral concentrations of IGF-I and diminished follicular development (Chase et al., 1998). It was speculated by the authors that low IGF-I levels led to inadequate LH receptor function or expression within granulosa cells. Therefore, reduced IGF-I levels would

lead to a diminished production of estradiol by granulosa cells.

Beam and Butler (1999) proposed the following model to explain the effects of IGF-I on reproductive recrudescence: immediately after parturition, there is a decrease in estradiol concentrations that allows for increased FSH secretion, which stimulates initial development of a new follicular wave between d 6 to d 8 postpartum. Both the initiation of a follicular wave and the selection of a dominant follicle are largely insensitive to early postpartum NEB. However, the steroidogenic activity and the acquirement of ovulatory competence of the dominant follicle are related to when postpartum energy balance begins to improve from its most negative level (i.e., nadir) and the associated increase in LH pulse frequency. The level of severity of NEB has a lesser modulating role on LH pulse frequency, but may influence circulating concentrations of insulin and IGF-I that are conductive to enhanced follicular cell responsiveness to the ongoing pulsatile LH secretion pattern. If the day of energy balance nadir occurs before or soon after the emergence of the dominant follicle, a concurrent increase in LH pulse frequency, as a consequence of metabolic signal at the hypothalamus, would promote differentiation of the dominant

follicle and increased estradiol production which would, ultimately, lead to the induction of an LH surge and first ovulation. Thus, according to the present model, IGF-I concentrations would function to increase responsiveness of the follicle to LH pulses, lead to a greater production of estradiol, and as a consequence, stimulate the occurrence of a preovulatory LH surge.

Alternatively, a delayed energy balance nadir would probably result in low LH pulse frequency, low production of estradiol, ovulation failure and atresia of the dominant follicle, or formation of follicular cysts. Reduced concentrations of IGF-I, and perhaps insulin, contribute to the reduced responsiveness to gonadotropic stimulation and to low estradiol production by the dominant follicle, exacerbating the effects of low pulsatile LH secretion. The fate of the first-wave dominant follicle affects the duration of the postpartum period of anestrus, with either the development of an anovulatory or cystic dominant follicle resulting in a greater number of days to first ovulation. However, the occurrence of an ovulation early in the puerperium period may not be beneficial to the reproductive performance of dairy cows. Early resumption of ovarian cyclicity after calving (< 19 d as compared to

>32 d) increased the risk for prolonged luteal cycles before service (Opsomer et al., 2000).

Alternatives for the Alleviation of Anestrus

Several different strategies can be adopted to minimize the incidence of prolonged periods of anestrus. These will be divided into management, nutritional, and pharmacological strategies as follows.

As discussed above, suckling is one of the most important factors leading to extended period of anestrus in beef cows. Therefore weaning of the calf is one management practice that can be adopted by cattle producers to increase reproductive performance of beef cows. Weaning can be partial, temporary, or complete. Partial weaning refers to the practice of separating the calves from their dams during most part of the day. Temporary weaning is when calves are completely removed for a period of usually 2 to 4 d. Both partial and temporary weaning can increase the number of cyclic cows during the breeding season, however, the response to these treatments is variable and management is somewhat difficult (Short et al., 1990). Alternatively, a more short-term solution may be complete weaning shortly before the beginning of the breeding season. Assuming that cows are in relative good body

condition and that calves are over 20 d old, most cows in anestrus are expected to resume cyclicity within 4 to 10 d (Short et al., 1990). However, early weaning is a costly, labor-intensive operation and may reduce growth rates of weaned calves (Commerford et al., 1995).

Energy balance is defined as the net energy intake of the animal minus the net energy required for maintenance and milk production (Mattos et al., 2000). Cows typically experience a period of negative energy balance after lactation and the extent of the period of negative energy balance was correlated with length of the anestrous period (Randel, 1990). Increased dry matter consumption can alleviate the NEB during early lactation. However, substantial increases in dry matter consumption usually are not attainable among high producing dairy cows. An alternative strategy is to increase the net energy of lactation diets by adding fat. Diets supplemented with fat have the potential to increase energy intake of cows when feed capacity may limit energy consumption shortly after parturition (Beam and Butler, 1998).

Indeed, supplemental fat is included frequently in rations to increase energy concentration in the diet to enhance energy intake and improve the energy status of the postpartum cow (Harrison et al., 1995; Palmquist and Weiss,

1994). Thus, it should be anticipated that, by increasing the energy status of lactating cows during the puerperium, recrudescence of ovarian function and reproductive performance also should be increased. However, in a review by Staples et al. (1998), it was observed that the inclusion of supplemental fat in the diet produced mixed results when reproductive performance was evaluated. Besides increasing the net energy content of diets, addition of fat had two other effects: it increased milk production and decreased dry matter consumption (Staples et al., 1998). Milk production was likely stimulated by an increased delivery of metabolizable energy when fat was supplemented (Andrew et al., 1991; Romo et al., 1996). A reduction in dry matter intake may be due to increased endogenous cholecystokinin (Choi et al., 1996) or by lowered acceptability of the fat-supplemented diet and increased rumen fill from reduced fermentation (Staples et al., 1998). Thus, the NEB is probably unaltered due to a decreased intake and increased milk output. Nonetheless, the observation that addition of fat to rations was able to enhance or inhibit fertility in cattle, even though a significant alleviation in the NEB may not have been obtained, suggests that fat may directly modulate the reproductive system in lactating cows.

Energy provided by fat supplementation increases LH secretion in animals that consume less energy than required (Hightshoe et al., 1991; Sklan et al., 1994). Supplemental fat also increased total number of follicles (Lucy et al., 1991; Wehrman et al., 1991; Beam and butler, 1997) and size of the preovulatory follicles (Lucy et al., 1990; Beam and Butler, 1997; Oldick et al., 1997). The increased size of the preovulatory follicle may be due in part to increased concentrations of plasma LH (Mattos et al., 2000). However, a mechanism independent from energy by which dietary fatty acids affect LH secretion has yet to be established.

Furthermore, specific fatty acids in the diet can modulate the production of $PGF_{2\alpha}$ by the uterine endometrium. $PGF_{2\alpha}$ is believed to participate in the process of uterine involution during the puerperium. The duration of increased $PGF_{2\alpha}$ production in the postpartum period is correlated negatively with the number of days to complete uterine involution and resumption of normal ovarian activity (Madej et al., 1984). Therefore, addition of specific fatty acids that may increase the production of $PGF_{2\alpha}$ may lead to a faster recovery from anestrus. Linoleic acid, which is one of the major fatty acids present in

soybean meal and whole cottonseed, is a precursor for arachdonic acid. Arachdonic acid is the fatty acid cleaved from the phospholipid membrane to yield $PGF_{2\alpha}$ (Mattos et al., 2000). Therefore, increased concentrations of linoleic acid may result in increased secretion of $PGF_{2\alpha}$ and a reduced period of anestrus. Infusion of lipid into ruminants increases plasma prostaglandins (Lucy et al., 1990; Burke et al., 1996; Filley et al., 1997). However, while feeding supplemental fat to favor increased linoleic acid in primiparous beef cows increased concentrations of prostaglandin $F_{2\alpha}$ metabolite in the early postpartum period, days to first estrus and ovulation and overall reproductive performance was not affected by diet (Filley et al., 2000). In contrast, feeding fatty acids that inhibit production of $PGF_{2\alpha}$ (i.e., linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid) during uterine involution may lead to increased length of anestrus.

The pharmacological use of progesterone (P_4) or progestins to induce estrus in postpartum anestrus cows has been well documented (Anderson et al., 1996; Favero et al., 1995; Fike et al., 1997 Miksch et al., 1978; Smith et al., 1987; Yavas et al., 1999). Maintaining circulating progestins at intermediate concentrations increases LH

pulse frequency (Bergfeld et al., 1996; Sanchez et al., 1995; Taylor et al., 1993). Thus, the effect of P_4 on the ovary involves increased LH secretion to cause an increase in follicular development and ovulation (Anderson et al., 1996; Imwalle et al., 1998). Nonetheless, the ability of P_4 treatment to overcome the inhibitory effects of anestrus may vary depending on the level of nutritional anestrus. Cows in a "deep" state of anestrus may not respond to P_4 administration with ovulation (Fike et al., 1997). Administration of estradiol benzoate after P_4 withdrawal increased the proportion of cows ovulating (Ulberg and Lindley, 1960; Saiduddin et al., 1968; Brown et al., 1972). Endogenous estradiol enhances the amplitude of LH pulses during the follicular phase of estrous cycles (Stumpf et al., 1989) by increasing the number of GnRH receptors at the anterior pituitary (Schoenemann et al., 1985). Thus, it appears that estradiol treatment after P_4 removal hastens or amplifies the preovulatory LH surge and expression of estrus in previous anestrous cows (Fike et al., 1997).

Treatment of anestrous cows with GnRH pulses during the postpartum period induced ovulation and CL formation (Walters et al., 1982; Jagger et al., 1987; Vizcarra et al., 1999), indicating that limited release of GnRH by the hypothalamus rather than the ability of the pituitary to

secrete LH is likely the principal cause of lactational anestrus. However, other investigators failed to observe an effect of intermittent GnRH injections on anestrous cows to induce ovulation (Short et al., 1981) and the lack of response was attributed to inadequate body condition. Thus, it seems that GnRH treatment may induce an ovulation in anestrous cows if anestrus is not too "deep" and there are follicles large enough on the ovary to respond to the induced LH surge.

As indicated above, concentrations of circulating IGF-I were correlated positively with resumption of ovarian activity (Beam and Butler, 1997; Burns et al., 1997; Roberts et al., 1997). Thus, treatment with low doses of bST may stimulate cows to cycle earlier during the postpartum period. Low doses of bST should not increase milk production and prolong the period of NEB, but may increase plasma concentrations of IGF-I and, consequently, induce ovarian recrudescence. When Stanisiewski et al. (1992) treated lactating cows with low doses of bST (5 mg/d) from 14 to 60 d postpartum, days to first postpartum estrus were reduced and first service conception rate was increased.

Another interesting approach to reducing postpartum anestrus is the pharmacological control of early follicular

development. Lewis et al. (1998) indicated that most cows develop a mild nonpathological endometritis during the early puerperal phase of the postpartum period. If the uterus is exposed to P_4 during this period as a result of an early postpartum ovulation, theoretically there is an increased risk for cows to develop uterine infections, because P_4 down-regulates the uterine immune system. Early resumption of ovarian cyclicity after calving (< 19 d as compared to >32 d) increased the risk for prolonged luteal cycles before service, which was associated positively with an increased incidence of metritis (Opsomer et al., 2000). Moreover, data from Beam and Butler (1997 and 1998) indicated that cows might develop ovarian cysts if development of a dominant follicle occurs early during the postpartum and becomes anovulatory due to NEB. Therefore, it is possible that resumption of a cyclic status early in the puerperium period and before alleviation of the NEB may prolong the period of anestrus and reduce fertility. If that is true, blockage of early follicular development presents the opportunity to reduce the incidence of puerperium disturbances (i.e., uterine infections and cystic ovaries) and potentially reduce the length of postpartum anestrus.

Mattos et al. (1998) indicated that administration of a GnRH agonist implant (Deslorelin; 2.1 mg) at 7 d postpartum temporally diminished early follicular development and reduced the cumulative increase of P_4 until 60 d postpartum. All deslorelin-treated cows ovulated to an injection of GnRH at 60 d postpartum and had similar subsequent plasma P_4 concentrations as compared to a control group, indicating that the effect on suppressed follicular development was temporary. Similarly, Haughian et al. (2000) injected cows with high doses of estradiol cypionate (ECP; 25 mg) at 7 d postpartum and completely eliminated the occurrence of ovulation prior to 30 d postpartum. Nonetheless, the percentage of cows with regular estrous cycles at 90 d postpartum was increased for ECP-treated cows. Therefore, the gonadotropin drive for follicular development was diminished temporally in both deslorelin and ECP-treated cows, which prevented ovulation and P_4 exposure during the period of uterine involution. These observations warrant further investigation towards the development of new management systems that improve subsequent reproductive efficiency of lactating dairy cows by delaying the first ovulation during the postpartum period.

Synchronization of Ovulation in Dairy Cattle

A large percentage of dairy producers have adopted the use of artificial insemination (AI) to breed heifers and lactating cows. The advantages that AI provided were responsible for its rapid acceptance since it became commercially available in the 1940's. The use of AI may eliminate the need for bulls within the milking herd, which constitute a liability for the safety of farm personnel, and also eliminates the transmission of sexually transmitted diseases within the herd. Most importantly, AI allows for a fast and accurate evaluation of the genetic merit of different sires (Odde et al., 1990). In this manner, only the best sires are actually used to disseminate superior production traits and that has enhanced greatly the rate of genetic improvement in dairy cattle. The use of AI also made accurate and efficient detection of estrus paramount for the success of reproductive management systems in dairy cattle. Therefore, this section of the literature review will focus on estrous synchronization systems with an emphasis on systems for dairy cattle that use timed artificial insemination.

The efficacy of reproductive management systems may be measured in different ways within dairy herds. Several

measurements such as days to first service, days to conception or days open, number of services per conception, calving interval, etc, have been used and constitute important tools to measure reproductive performance. Nonetheless, pregnancy rate is believed by many to be the single most important factor to describe success or failure of a reproductive management system. Pregnancy rate measures the percentage of cows that become pregnant after insemination among the total number of cows eligible for breeding within a period of time (usually 21 d, which corresponds to the average length of the estrous cycle). It is important to specifically define what are the factors that influence pregnancy rate.

Estrous detection rate is defined as the percentage of cows that are observed expressing behavioral signs of estrus among all cows eligible for breeding. Conception rate measures the percentage of cows diagnosed pregnant after AI among cows detected in estrus and inseminated. In this manner, the product of estrous detection rate and conception rate is pregnancy rate (Macmillan, 1992). Thus, it is easy to understand the impact that estrous detection has on pregnancy rates. In 1974, Barr reported that increased calving intervals had a .92 correlation with failure to observe estrus, whereas only a .38 correlation

was detected between calving intervals and conception rates. Furthermore, Ferguson and Galligan (1993) observed that 79% of the variation in calving intervals is explained by first-service pregnancy rates, which indicates that detection of estrus in the early postpartum period is of paramount importance and greatly affects the overall herd reproductive performance.

In order to improve estrous detection rates, several systems that synchronize estrus have been developed. Estrous synchronization allows cattle managers to concentrate the effort of detecting estrous behavior to a relatively short period of time. Thus, the main purpose for estrous synchronization is to facilitate the use of AI (Barber, 1993). Ideally, an estrous synchronization system should elicit a fertile, tightly synchronized estrus response in a high percentage of treated females (Odde, 1990).

Since 1951, estrus has been synchronized through the use of progesterone or progestins compounds (Ulberg et al., 1951) that avoided ovulation of a dominant follicle while allowing for the spontaneous regression of the CL and for the decrease of plasma concentrations of endogenous progesterone. Hence, cows would only express estrus and ovulate a few days after removal of the exogenous source of

progesterone. Progesterone and progestin compounds were developed and several delivery systems ranging from daily injections, intravaginal releasing devices, subcutaneous implants, to food additives have been tested (Macmillan and Peterson, 1993; Roche, 1974; Wiltbank et al., 1965). Such systems have the disadvantage of developing persistent follicles that have reduced fertility after ovulation (Savio et al., 1993b; Smith and Stevenson, 1995) because follicular development is not synchronized. Such a decrease in fertility of persistent follicles may be associated with a prolonged exposure to high concentrations of estradiol and to a high pulsatile secretion of LH (Austin et al., 1999). Follicular development can be synchronized with estrogens (Bo et al., 1995), and the use of estradiol at initiation of progestin treatment to synchronize follicular development is currently under intensive investigation (Cavalieri et al., 1997; Day et al., 2000; Yelich et al., 1997). Nonetheless, the use of steroid hormones (with the sole exception of estradiol cypionate), which includes progesterone and progestins, is not allowed for lactating dairy cows, which limits their importance for the dairy industry.

Other estrous synchronization systems have taken advantage of the discovery of the luteolysin prostaglandin

$F_{2\alpha}$ ($PGF_{2\alpha}$) to allow for the control of the lifespan of the CL. Administration of $PGF_{2\alpha}$ induces CL regression and allows for final development and maturation of the dominant follicle present at the ovary. Such effects of $PGF_{2\alpha}$ have been reviewed extensively (Inskeep et al., 1973; Thatcher and Chenault, 1976). However, it was observed that luteolysis cannot be induced during the first 5 d of the estrous cycle (Lauderdale, 1972; Hafs et al., 1974), although receptors for $PGF_{2\alpha}$ are present on the CL 2 d after ovulation (Wiltbank et al., 1995). This lack of responsiveness was associated with an uncoupling of receptors and second messenger systems that leads to failure of induction of prostaglandin synthase-2 and to an incomplete differentiation of the degenerative mechanisms in newly differentiated luteal cells (Wiltbank et al., 1995; Tsai and Wiltbank, 1998). Furthermore, during the proestrous phase of the estrous cycle, which occurs after endogenous release of uterine $PGF_{2\alpha}$, administration of $PGF_{2\alpha}$ also has no effect. Therefore, use of $PGF_{2\alpha}$ is only effective if injected between Days 6 to 17 of the estrous cycle.

Theoretically, within a group of cows, which are cycling and equally distributed among the different days of

the estrous cycle, a single injection of $PGF_{2\alpha}$ should induce estrus in 75% of the animals. The use of two consecutive injections of $PGF_{2\alpha}$ within an 11-d interval in heifers (Lauderdale et al., 1981; Van Cleeff et al., 1991) or within a 14-d interval in cows (Folman et al., 1990; Ferguson and Galligan, 1993) increases the percentage of animals observed in estrus after the second injection of $PGF_{2\alpha}$.

Estrus induced by $PGF_{2\alpha}$ is expressed within a 7 d period. The induction of estrus during a 7 d period is due to different stages of follicular development at the injection of $PGF_{2\alpha}$ (Kastelic et al., 1990; Savio et al., 1990). Estrus was more precisely synchronized (3 d after $PGF_{2\alpha}$ injection) if $PGF_{2\alpha}$ was administered on Days 7, 15 and 16 of the cycle in comparison to cows injected on Days 8 to 14 of the cycle, in which estrus occurred between 4 to 7 d after $PGF_{2\alpha}$ administration (Macmillan and Henderson, 1984). Interpretation of this and similar observations (Tanabe and Hahn, 1984; Watts and Fuquay, 1985; Stevenson et al., 1998) indicate that if a dominant follicle is present at the time of induced luteolysis, rapid maturation occurs and estrus is expressed shortly (i.e., 2 to 3 d) after $PGF_{2\alpha}$ injection. In contrast, if a follicular recruitment is occurring and

the dominant follicle is not present at the time of $PGF_{2\alpha}$ injection, several additional days may be necessary for the selection and maturation of a new dominant follicle. Thus, the synchrony of estrus induced by $PGF_{2\alpha}$ is not precise because follicular development is not synchronized and results in a greater period of time devoted to estrous detection.

Synchronization of Follicular Development

Ovarian follicular development has been well characterized after the development of real time ultrasound imaging and the development of rectal probes for use in cattle (Pierson and Ginther, 1984). When ultrasonography was used to follow the pattern of development of follicles that are greater than 5 mm in diameter, the existence of two to three periods of follicular development within an estrous cycle was demonstrated (Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989). Each period of follicular development that culminated in the development of a single large follicle was designated as a follicular wave characterized by three phases. Initially, a group of 3 to 6 small follicles starts to grow and reach about 5 mm in diameter during the recruitment phase. After recruitment, the selection phase starts during which only

one follicle continues to grow and becomes larger than the other follicles. After selection, the selected follicle continues to grow until it enters a plateau phase during the dominance phase. Throughout the dominance phase, the dominant follicle secretes estrogen and inhibits the growth of other follicles on both ipsi- and contralateral ovaries. Because estrus results from CL regression coordinated with the growth of an estrogenic dominant follicle (Chenault et al., 1975; Thatcher and Chenault, 1976), control of follicular development is important to obtain a tightly synchronized expression of estrus.

Follicular development may be synchronized in different ways. Ablation of the dominant follicle by electrocauterization or by transvaginal aspiration of the dominant follicle guided by ultrasonography results in the recruitment of a new follicular wave (Adams et al., 1992a; Wolfsdorf et al., 1997), but such methods have limited practical value. Progesterone or estradiol treatment may also be used to induce follicular turnover and synchronize the emergence of a new follicular wave (Adams et al., 1992b; Adams et al., 1994; Bo et al., 1995). However, as stated above, use of steroidal drugs is not approved for lactating dairy cattle and limited data are available on the use of steroid hormones to regulate follicular waves in

dairy cows. Another method consists of injecting gonadotropin-releasing hormone (GnRH) or its agonists. Injection of GnRH or its agonists induces the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Chenault et al., 1990). The induced surge of LH causes ovulation or luteinization of large ovarian follicles present at the time of treatment and initiates the recruitment of a new follicular wave (Thatcher et al., 1989; Macmillan and Thatcher, 1991; Twagiramungu et al., 1994). Therefore, administration of GnRH efficiently synchronizes follicular development in cattle.

The use of GnRH to synchronize follicular development, followed 7 d later with an injection of $PGF_{2\alpha}$ to induce luteolysis, was described as an estrous synchronization method by Thatcher et al. (1989), and then by Macmillan and Thatcher (1991). Results indicated that estrous detection rates were increased compared to those obtained after a single injection of $PGF_{2\alpha}$, and no differences in conception rates between the two groups were observed. In addition, the precision of synchrony also was enhanced when cows were injected with GnRH 7 d prior to the injection of $PGF_{2\alpha}$, with most estruses being detected 2 to 3 d after $PGF_{2\alpha}$

administration. These results were later confirmed in subsequent experiments, where it was concluded that the use of GnRH followed 6 or 7 d later with $PGF_{2\alpha}$ is a satisfactory system for synchronization of estrus with good fertility (Coleman et al., 1991; Guilbault et al., 1991; Twagiramungu et al., 1995).

The Ovsynch/TAI Protocol

Because the use of GnRH and $PGF_{2\alpha}$ 7 d later induced a tightly synchronized expression of estrus, experiments were designed to test whether estrus and ovulation could be precisely controlled to allow for AI at a fixed time. An insemination performed at a fixed time would eliminate the labor associated with estrous detection and also management constraints and inefficiencies of such an activity. Moreover, such a technology would provide dairymen with an opportunity to increase pregnancy rates, because 100% of cows eligible for breeding would be presented for insemination.

In 1995, Pursley et al. described a protocol consisting of an injection of GnRH followed 7 d later with an injection of $PGF_{2\alpha}$ and a second injection of GnRH 48 h later. After the second injection of GnRH, the ovaries were scanned continuously (every 2 to 4 h) starting 20 h

after injection of GnRH. Ovulation of the dominant follicle was detected 26 to 32 h after the second injection of GnRH in all synchronized cows (20/20). This evidence suggested that the time of ovulation was tightly synchronized after the protocol, which is referred to as the Ovsynch/TAI protocol. Indeed, ovulation was synchronized to a degree that would allow for a timed insemination without the need for estrus detection.

After these observations, several experiments were performed comparing pregnancy rates between cows submitted to the Ovsynch/TAI protocol to pregnancy rates after several other estrous synchronization treatments in which cows were inseminated at detected estrus. Results from such experiments are depicted in Table 2.1. Estrous synchronization systems described in Table 2.1 varied from a single injection of $PGF_{2\alpha}$ to two or more $PGF_{2\alpha}$ injections followed by timed insemination (i.e. target breeding system) with or without follicular synchronization induced by GnRH. Other investigators compared pregnancy rates to the Ovsynch/TAI protocol to pregnancy rates after cows were injected with GnRH, $PGF_{2\alpha}$ 7 d later, and inseminated at observed estrus.

Table 2.1. Pregnancy rates (PR) of lactating cows submitted to the Ovsynch/TAI protocol (TAI) as compared to other breeding systems based on insemination at detected estrus (IDE).

Reference	Control group	n	PR (%)[1] IDE	PR (%)[1] TAI[10]	P
Burke et al., 1996	GnRH+PGF [2]	299	30.5	29.0	NS[11]
Stevenson et al., 1996	2xPGF [3]	236	26.5	35.3	NS
Pursley et al., 1997a	3xPGF+TAI [4]	310	38.9	37.8	NS
Pursley et al., 1997b					
- First service	PGF [5]	298	39.0	37.0	NS
- Second service	PGF [5]	163	45.0	42.0	NS
- Third service	PGF [5]	83	61.0	48.0	NS
Momcilovic et al., 1998	AI at estrus [6]	183	6.0	33.0	.01
	PGF [7]	247	12.0	33.0	.01
	2xPGF	180	11.0	33.0	.01
Stevenson et al., 1999					
- Experiment 1	GnRH+PGF	142	25.6	22.1	NS
	2xPGF+GnRH+TAI [8]	133	24.6	22.1	NS
	2xPGF+TAI [9]	169	31.7	22.1	NS
- Experiment 2	GnRH+PGF	227	26.8	35.6	NS

[1] Pregnancy diagnosis was performed from 25 to 52 d after AI.

[2] GnRH, $PGF_{2\alpha}$ 7 d later, and cows were AI at estrus.

[3] Two $PGF_{2\alpha}$ given 14 d apart and cows were AI at estrus.

[4] Three $PGF_{2\alpha}$ and AI at estrus. Cows not observed in estrus were TAI at 72 to 80 h after the third $PGF_{2\alpha}$ injection.

[5] Cows eventually received $PGF_{2\alpha}$ and were AI at estrus.

[6] Cows received no treatments and were AI at detected estrus.

[7] Cows received a single $PGF_{2\alpha}$ and were AI at detected estrus.

[8] Two $PGF_{2\alpha}$ given 14 d apart, GnRH given 33 h after second $PGF_{2\alpha}$, and cows were TAI 16 to 18 h after injection of GnRH.

[9] Two $PGF_{2\alpha}$ given 14 d apart and cows were AI at estrus. Cows not observed in estrus were TAI 80 h after the second $PGF_{2\alpha}$ injection.

[10] The time between the $PGF_{2\alpha}$ and the second GnRH and the time between the second GnRH and the TAI was slightly modified from the Ovsynch/TAI protocol described above in some of the experiments.

[11] NS = Not significant.

Collectively, these data suggest that pregnancy rates obtained with the Ovsynch/TAI protocol were similar to pregnancy rates to several estrous synchronization methods. Pregnancy rates to the Ovsynch/TAI protocol were greater than for control groups in one experiment (Momcilovic et al., 1998), but pregnancy and estrous detection rates for the control groups were low, partially due to an effect of season in that particular experiment. It is important to note that estrous detection rates for the experiments described above (range = 55.5 to 82.3%) were considerably higher than the average estrous detection rate reported for the US (approximately 50%). It should be expected that in cases where estrous detection rates are high, differences in pregnancy rates between controls and the Ovsynch/TAI protocol would be difficult to detect.

Moreover, the Ovsynch/TAI protocol did not seem to affect fertility to subsequent services. As described in Table 1, Burke et al. (1996) observed no differences in first service pregnancy rates of cows submitted to the Ovsynch/TAI or control cows. After the first synchronized service, cows assigned to the Ovsynch/TAI group were re-inseminated at detected estrus, as were control cows. Differences in pregnancy rates at 120 d postpartum would have indicated differences in fertility to subsequent

services. However, such differences were not observed and no differences were observed in pregnancy rates at 120 d postpartum (control = 58.8% and Ovsynch/TAI = 56.2%), which indicate that fertility for second and subsequent services were similar for both groups. Similarly, no differences in pregnancy and culling rates at the end of lactation were observed between cows receiving Ovsynch/TAI at first service and control cows (Stevenson et al., 1996 and 1999). The interval from the synchronized service to the second service of Ovsynch/TAI cows did not differ compared to control cows (33.1 d and 37.0 d, respectively). Also mean intervals of short (< 17 d), normal (17 to 25 d), or long (> 25 d) return to estrus after insemination were not affected by treatment (Stevenson et al., 1996). Nonetheless, in a study involving dairy heifers, Schmitt et al. (1996b) observed that the incidence of short return to estrus after insemination (< 17 d) was greater for the Ovsynch/TAI group (15.5%, 29/187) than for the control group (4.3%, 6/139). This effect was not observed when the second GnRH injection was substituted by an injection of hCG in that no differences in the incidence of short estrous returns were detected (Ovsynch + hCG/TAI = 6.0%, 6/102 and control = 1.0%, 1/96; Schmitt et al., 1996).

The incidence of double ovulations after the second GnRH injection of the Ovsynch/TAI protocol was 14.1% (28/199; Fricke et al., 1998). Previous reports in the literature have indicated that incidence of double ovulations after a spontaneous estrus varied from 13.1% (Kidder et al., 1952) to 5.4% (Labhsetwar et al., 1963). However, such results were based on palpation of CL 7 to 13 d after estrus, which may underestimate the incidence of double ovulations. Interestingly, high milk production was associated with increased rate of double ovulations (Fricke and Wiltbank, 1999). Moreover, pregnancy rates for cows successfully synchronized after the Ovsynch/TAI protocol were increased if cows had a double ovulation (64.0%, 16/25) than if cows had a single ovulation (45.2%, 75/166) after the second GnRH injection (Fricke and Wiltbank, 1999). The incidence of twins after the Ovsynch/TAI protocol was 2.4% (18/732) and 5.2% (3/58) in two previous studies (Pursley et al., 1998 and Fricke and Wiltbank, 1999, respectively). A recent report, in which records of over 52,000 lactations across 10 years were analyzed, observed a twinning rate of approximately 2.5% (Kinsel et al., 1998). Such results were within the normal range from 1 to 5% previously observed (Sreenan and Diskin, 1989).

Thus, the incidence of twins does not seem to increase after the Ovsynch/TAI protocol.

No studies have tested whether the incidence of ovarian cysts was increased after the Ovsynch/TAI protocol. However, such an effect should not be expected, because the use of GnRH and $PGF_{2\alpha}$ has been prescribed as a treatment for cystic cows (Archbald et al., 1991). Indeed, among cows diagnosed as cystic, pregnancy rates to the Ovsynch/TAI protocol (23.6%, 18/76) were similar to pregnancy rates of a control group, which received GnRH followed 7 d later by $PGF_{2\alpha}$ and cows were inseminated at detected estrus (18.0%, 15/83; Bartolome et al., 2000). Fricke et al. (1999) observed that, among cows diagnosed as cystic, 73.1% (19/26) ovulated a follicle other than the cystic follicle after the second GnRH injection of the Ovsynch/TAI protocol and 36.8% (7/19) of the synchronized cows conceived to the timed insemination.

Therefore, the use of the Ovsynch/TAI protocol resulted in similar pregnancy rates compared to several estrous synchronization methods and did not seem to increase the incidence of reproductive problems (i.e., fertility to subsequent services, twining rates, cystic ovaries) after the synchronized service. However, in many of the studies described above, conception rates to the

Ovsynch/TAI protocol were lower when compared to control groups that depended upon estrous detection (Burke et al., 1996; Stevenson et al., 1996; Pursley et al., 1997a; Stevenson et al., 1999). This was an important observation because it indicated that there are problems associated with the Ovsynch/TAI protocol that do not result in similar conception to an artificial insemination. It can be argued that, when cows undergo the Ovsynch/TAI protocol, there are no distinctions between cyclic, fertile cows from cows in anestrus or with reproductive problems (i.e., uterine infections, cystic ovaries, oviduct adhesions, etc) and all cows are inseminated. That does not occur in estrous synchronization systems because cows in anestrus and with reproductive problems frequently are not observed in estrus and, therefore, are not inseminated. Nonetheless, inefficiencies inherent to the Ovsynch/TAI protocol may also have an important role in reducing conception rates. Understanding the physiological mechanisms by which the Ovsynch/TAI protocol affects ovarian development may allow us to correct inefficiencies associated with the program, produce significant gains in terms of conception rates, and result in similar conception rates between cows submitted to the Ovsynch/TAI protocol and cows inseminated at detected estrus.

Theoretically, the first injection of GnRH of the Ovsynch/TAI protocol should either ovulate or luteinize any large follicles present on the ovaries and, as a consequence, tightly synchronize the emergence of a new follicular wave. Then, 7 d later, the injection of $PGF_{2\alpha}$ should induce complete CL regression and the levels of plasma P_4 should decrease to less than 1.0 ng/mL within 48 h, allowing for the final maturation of the dominant follicle whose emergence was synchronized 7 d earlier. Finally, the second injection of GnRH should induce a tightly synchronized ovulation approximately 28 h later. However, researchers observed that such an ideal scenario does not always occur. Indeed, it was hypothesized that the stage of the cycle at which the Ovsynch/TAI protocol is initiated has a profound effect on ovarian dynamics and subsequent pregnancy rates.

Effect of Stage of the Estrous Cycle at Initiation of the Ovsynch/TAI Protocol

Pursley et al. (1995) reported that ovulation rates to the first GnRH injection given at random stages of the estrous cycle were 90.0% (18/20) in cows and 54.1% (13/24) in heifers. Such a lower response to the first GnRH injection in heifers was suggested later as a cause for unsatisfactory fertility in heifers after the Ovsynch/TAI

program (Pursley et al., 1997a). In a subsequent experiment involving a greater number of cows (n = 156), the overall ovulation rate to the first GnRH injection was 64% (Vasconcelos et al., 1999b). In this same study, day of the estrous cycle at which the first GnRH injection was administered affected ovulation rates. When GnRH was injected between Days 1 to 4 (n = 31), 5 to 9 (n = 47), 10 to 16 (n = 52), and 17 to 21 (n = 26), ovulation rates were 23%, 96%, 54%, and 77%, respectively. Similarly, for the small number of heifers which received the first GnRH injection at d 2, 5, 10, 15, and 18 of the estrous cycle, ovulation rates were 0% (0/5), 100% (5/5), 25% (1/4), 60% (3/5), and 100% (5/5), respectively (Moreira et al., 2000b).

Results from the last two studies reflect the influence of stage of the estrous cycle on the capacity of the first GnRH injection of the Ovsynch/TAI protocol to synchronize follicular development. During the metestrous period (i.e., d 1 to 4 of the cycle), there are probably no large follicles present in the ovaries that are able to respond to the first injection of GnRH, which explains why low ovulation rates were observed. In contrast, high ovulation rates were observed during the early luteal phase, which corresponds to the period of dominance of the first-wave

follicle (i.e., d 5 to 9 of the cycle). It has been well documented that the first follicular wave is the most predictable wave and that administration of GnRH at this stage would most likely result in ovulation (Schmitt et al., 1996a, Diaz et al., 1998). During the late luteal phase (i.e., Days 10 to 17 of the cycle), after the first-wave dominant follicle undergoes atresia and a new follicular wave emerges, the pattern of follicular development is quite variable, depending upon the occurrence of two or three follicular waves. Therefore, ovulation rates to an injection of GnRH at the late luteal phase predictably should be higher than ovulation rates obtained at metestrus, but also lower than ovulation rates after a GnRH injection during the early luteal phase. Finally, during the proestrous period (i.e., d 18 to 21 of the cycle), there is a high probability that a dominant follicle, which would become the ovulatory follicle, is present in the ovaries. Thus, as observed, ovulation rates to an injection of GnRH are high if cows are injected during the proestrous phase.

As indicated before, one of the objectives of the Ovsynch/TAI protocol is to coordinate emergence of a new follicular wave with the onset of luteal regression. Upon a GnRH-induced ovulation, such a tight synchrony in the

emergence of a new follicular wave is obtained and a new dominant follicle is selected approximately 2 to 3 d after the administration of GnRH (Pursley et al., 1995; Moreira et al., 2000b). However, such a high degree of synchrony may not be obtained if cows fail to ovulate to the first injection of GnRH. Indeed, cows do not necessarily need to ovulate a follicle to have a synchronized emergence of a new follicular wave. If a dominant follicle is present at the time of GnRH administration and it undergoes luteinization instead of ovulation, the emergence of a synchronized new follicular wave is still obtained (Moreira et al., 2000b). Another possible scenario in which follicular synchronization may occur without the need for ovulation is if, by chance, a new follicular wave emerges spontaneously 2 to 3 d after the GnRH injection. Such a scenario may occur if a cow initiates the Ovsynch/TAI protocol at the early metestrus, shortly after a normal endogenous ovulation, or during the diestrus phase, shortly after the interval between two follicular waves.

However, because follicular development in cases like the latter two is not controlled, the emergence of a new dominant follicle may not be synchronized and occur as early as the day prior to or the day after the first GnRH injection of the Ovsynch/TAI protocol (Pursley et al.,

1995; Moreira et al., 2000b). Such an unsynchronized dominant follicle is a potentially aged follicle that reaches 10 mm in diameter and remains in the ovary for a greater period of time prior to ovulation (Moreira et al., 2000b). Presence of such an aged follicle at the GnRH-induced ovulation may compromise subsequent fertility of the Ovsynch/TAI protocol by three mechanisms: ovulation failure after the second GnRH injection, induction of a less robust CL after ovulation, and ovulation of a less fertile oocyte.

The number of LH receptors decreases as the dominant follicle develops from growth into the plateau and regression phases (Rollosson et al., 1994) and as atresia clearly is manifested (Guibault et al., 1993). Indeed, Silcox et al. (1993) observed that GnRH treatment alters the diameter of the dominant follicle during its growing or plateau phase, but not during its regression phase. Thus, the presence of an aged follicle at the time of administration of the second GnRH injection of the Ovsynch/TAI may increase the chances for an ovulation failure.

Pursley et al. (1995) observed that synchronization rates after the second GnRH was 100% (20/20) among cows and 75% (18/24) among heifers. The lower synchronization rate

observed in heifers was attributed to the lower frequency of heifers that ovulated a follicle after the first GnRH injection and failed to synchronize emergence of a new follicular wave (Pursley et al., 1995). Moreira et al. (2000b) observed that dominant follicles that failed to ovulate to a second GnRH injection of the Ovsynch/TAI protocol remained greater than 10 mm in diameter for a longer period than dominant follicles that ovulated to the second GnRH injection (7.5 d > 5.0 d). However, the small number of observations in the previous analyses limits the scope of these results. Fricke and Wiltbank (1999) reported that ovulation to the second injection of GnRH was synchronized in 84% of cows after an Ovsynch/TAI service. Ovulation failure after the second injection of GnRH occurred in 7 .0% (11/156) of cows that had undergone the Ovsynch/TAI protocol (Vasconcelos et al., 1999b). However, ovulation failure to the second GnRH injection was not associated with stage of the cycle at initiation of the Ovsynch/TAI program or with ovulation failure to the first GnRH injection.

It was reported that plasma P_4 concentrations were reduced upon ovulation of aged follicles with prolonged period of dominance (Moreira et al., 2000b). This may indicate that such a follicle was affected by the prolonged

dominance period and subsequent CL development was impaired. Because the increase in plasma P_4 concentration after insemination was positively correlated to pregnancy rates (Maurer and Echternkamp, 1982; Butler et al., 1996), ovulation of such an aged follicle may compromise subsequent fertility to the synchronized service.

Prolonged periods of follicular dominance prior to ovulation reduce subsequent pregnancy rates (Austin et al., 1999). The latter authors contended that such a reduction in fertility of these aged follicles is probably due to a prolonged exposure of the dominant follicle to the effects of estradiol. Extended intrafollicular concentrations of estradiol, along with increased LH pulsatility, may perturb the normal temporal relationship between oocyte maturation and preovulatory maturation of the follicle. This scenario results in the ovulation of excessively aged oocytes that compromise subsequent fertility (Austin et al., 1999).

Collectively, these observations indicate that a tight synchronization of follicular development is paramount for the subsequent fertility to an Ovsynch/TAI service, and that initiation of the Ovsynch/TAI protocol during the metestrus period decreases the probability of inducing a synchronized emergence of a new follicular wave.

Since the initial studies involving estrous synchronization based on GnRH and $PGF_{2\alpha}$ were described, it has been observed that cows may express estrus prior to the injection of $PGF_{2\alpha}$. Thatcher et al. (1989) reported that 10.3% (21/203) of heifers injected with GnRH at d 0 were observed in estrus prior to the injection of $PGF_{2\alpha}$ 7 d later. Similarly, Twagiramungu et al. (1992) reported that cows injected with GnRH were observed in estrus prior to an injection of $PGF_{2\alpha}$ 6 d later, although the incidence of estrus within 6 d of the administration of GnRH was reduced compared to controls. In two experiments using dairy heifers, Schmitt et al. (1996) indicated that 25.1% (experiment 1; 47/187) and 22.5% (experiment 2; 23/102) of the experimental animals were observed in estrus less than 39 h after the injection of $PGF_{2\alpha}$ of the Ovsynch/TAI protocol. Data from Burke et al. (1996) indicated that 17.3% (52/299) of experimental cows submitted to the Ovsynch/TAI protocol or to a control group (GnRH followed by $PGF_{2\alpha}$ 7 d later) had plasma P_4 concentrations below 1.0 ng/mL at the time of injection of $PGF_{2\alpha}$. Although some of the latter cows may have been anestrous cows, it is possible that some cows were not anestrus but regressed their CL prior to the injection of $PGF_{2\alpha}$, and such cows were

probably in estrus prior to or immediately after the day of $PGF_{2\alpha}$ administration. Among cows assigned to the Ovsynch/TAI group, approximately 10.0% (17/171) were detected in estrus prior to the second GnRH injection of the synchronization protocol.

Similarly, Stevenson et al. (1996) observed that 17.6% (15/85) of cows were in the follicular phase (i.e., plasma P_4 < 1.0 ng/mL) at the time of $PGF_{2\alpha}$ injection preceded 7 d earlier by an injection of GnRH. It was reported that 13.8% (16/116) of cows had plasma P_4 concentrations less than 1.0 ng/mL at the time of $PGF_{2\alpha}$ injection, but pregnancy rates for such cows were similar to pregnancy rates of cows that had plasma P_4 concentrations greater than 1.0 ng/mL (43.8%, 7/16; and 37.0%, 37/100; respectively; Pursley et al., 1997). Nonetheless, the previous study also reported that 40.5% (30/74) of heifers undergoing the Ovsynch/TAI protocol had plasma P_4 concentrations lower than 1.0 ng/mL at the time of injection of $PGF_{2\alpha}$, and pregnancy rates for the latter heifers were reduced when compared to pregnancy rates of heifers that had plasma P_4 concentrations greater than 1.0 ng/mL at $PGF_{2\alpha}$ injection (20.0%, 6/30 < 47.7%, 21/44). Stevenson et al. (1999) indicated that 24.2% of cows in experiment 1 (31/128) had plasma P_4 lower than 1.0

ng/mL at the time of $PGF_{2\alpha}$ injection, 7 d after an injection of GnRH. It was reported that only 3% of all experimental cows were anestrus in this experiment, which implies that most of the cows with low plasma P_4 concentrations at $PGF_{2\alpha}$ injection were probably cyclic cows that regressed their CL prior to the injection of $PGF_{2\alpha}$. Furthermore, in experiment 2, Stevenson et al. (1999) reported that 17.9% of cows (34/190) had plasma P_4 concentrations greater or equal to 1.0 ng/mL at injection of GnRH (HIGH) but had plasma P_4 concentrations of less than 1.0 ng/mL 7 d later at the time of $PGF_{2\alpha}$ administration (LOW). Moreover, pregnancy rates for such a group of cows (HIGH-LOW; 28.6%; 6/21) were numerically lower than for cows with low P_4 concentrations at the GnRH injection and high P_4 concentrations at $PGF_{2\alpha}$ 7 d later (LOW-HIGH; 40.0%; 12/30) or cows that had high plasma P_4 concentrations at both samples (HIGH-HIGH; 36.7%; 22/60) when cows were timed inseminated. Authors argued that no differences were detected possibly because of an insufficient number of observations (Stevenson et al., 1999). However, when HIGH-LOW cows were inseminated at detected estrus after the injection of $PGF_{2\alpha}$, pregnancy rates for HIGH-LOW cows were similar to pregnancy rates of LOW-HIGH and HIGH-HIGH cows (38.5%, 5/13; 43.4%, 10/23; and

42.1%, 16/38; respectively). Thus, it seems that fertility of HIGH-LOW cows is impaired only if they are timed inseminated. Such data agreed with subsequent observations by Moreira et al. (2000d). In the latter study, it was observed that approximately 6.0% of all cyclic cows (18/303) were classified as HIGH-LOW, and that pregnancy rates to a timed insemination for HIGH-LOW cows were reduced compared to pregnancy rates for LOW-HIGH and HIGH-HIGH cows (12.6% < 27.8% and 38.4%, respectively).

Collectively, results suggest that pregnancy rates are reduced after a timed insemination service of cyclic cows that prematurely regress their CL prior to the injection of $PGF_{2\alpha}$ of the Ovsynch/TAI protocol. Thus, it was necessary to determine the cause for such a premature demise of the CL. Vasconcelos et al. (1999b) observed that among 156 cows submitted to the Ovsynch/TAI protocol, 9 cows (approximately 6%) ovulated a dominant follicle prior to the second GnRH injection of the timed insemination program. All cows having premature CL regression had initiated the Ovsynch/TAI protocol at later stages of the estrous cycle (i.e., d 13 to d 22 of the cycle) and had not ovulated to the first injection of GnRH. In a second experiment, it was observed that 20% of the cows (25/125) had low plasma P_4 concentrations at the time of $PGF_{2\alpha}$

injection and that, among those, 32% (8/25) ovulated before the second injection of GnRH. Once again, it was observed that cows initiating the Ovsynch/TAI protocol at later stages of the estrous cycle (i.e., between d 10 and d 21 of the cycle) were more likely to have low plasma P_4 at $PGF_{2\alpha}$ injection. Thus, the reason for low pregnancy rates of HIGH-LOW cows is probably due to an asynchrony of ovulation because cows that ovulate prior to the second GnRH injection are at lower odds to conceive to a timed insemination service. Moreover, the incidence of premature CL regression and asynchrony of ovulation seems to be related to the stage of the estrous cycle at which the Ovsynch/TAI protocol is initiated.

Moreira et al. (2000b) conducted an intensive study of follicular and CL development during the Ovsynch/TAI protocol, at which daily scanning of ovaries and daily collection of blood samples from heifers pre-synchronized to initiate the Ovsynch/TAI protocol at different stages of the cycle was performed. Results indicated that in all heifers which initiated the timed insemination program at d 15 of the estrous cycle, and only in that particular group, premature CL regression (as determined by ultrasonography and plasma P_4 concentrations), and expression of estrous behavior was observed prior to the second GnRH injection of

the Ovsynch/TAI protocol (5/5). Among these heifers, 60% (3/5) ovulated prior to the second GnRH injection. Interestingly, complete CL regression, characterized by plasma $P_4 \leq 1.0$ ng/mL at the day of $PGF_{2\alpha}$ injection, was observed in heifers that had ovulated (3/3) or not (2/2) to the first injection of GnRH administered 7 d earlier. Such an observation agrees with previous data (Howard and Britt, 1990) indicating that, among heifers in which a accessory CL was induced at d 10 of the cycle, regression of both endogenous and accessory CL was induced by an injection of $PGF_{2\alpha}$ at 2, 4, or 6 d later. It was concluded that an accessory CL induced within a period of high P_4 concentrations was able to respond to an injection of $PGF_{2\alpha}$ at earlier stages of development. Thus, premature CL regression during the Ovsynch/TAI protocol was associated with initiation of the Ovsynch/TAI protocol at the late luteal phase (i.e., d 15 of the cycle).

After follicular synchronization, the second main event that needs to occur within the Ovsynch/TAI protocol is the demise of original and the GnRH-induced CL. However, complete CL regression does not always follow injection of $PGF_{2\alpha}$. Although the initial study by Pursley et al. (1995) indicated that all cows (20/20) submitted to the

Ovsynch/TAI protocol regressed their CL after the injection of $PGF_{2\alpha}$, others reported different results. Schmitt et al. (1996) determined that 20.4% (37/181) of heifers submitted to the Ovsynch/TAI protocol had plasma P_4 concentrations greater than 2.0 ng/mL 48 h after $PGF_{2\alpha}$ administration. In the experiment performed by Burke et al. (1996), 8.3% of the cows (4/48) had plasma P_4 concentrations greater than 1.0 ng/mL 48 h after the injection of $PGF_{2\alpha}$. None of the cows that showed signs of incomplete CL regression conceived to the Ovsynch/TAI service. Stevenson et al. (1999) reported that 15.2 to 24.7% of cows had plasma P_4 concentrations equal to or greater than 1.0 ng/mL 24 h after injection of $PGF_{2\alpha}$. In subsequent experiments, Pursley et al. (1997) also observed that cows sometimes fail to undergo complete CL regression and reported that 6.0% of cows (7/116) and 4.0% of heifers (3/77) had plasma P_4 concentrations greater than 1.0 ng/mL 24 to 48 h after the injection of $PGF_{2\alpha}$. The magnitude of the incidence of incomplete CL regression was greater in an experiment of Moreira et al. (2000d) where approximately 19.0% (62/327) of cows failed to completely regress their CL, as determined by plasma P_4 concentrations greater than 1.0 ng/mL 48 h after the injection of $PGF_{2\alpha}$. Moreover,

pregnancy rates for the latter group of cows were reduced compared to pregnancy rates of cows that completely regressed their CL (8.4% < 35.0%; P < 0.01). Hence, the frequency of cows failing to undergo complete CL regression after $PGF_{2\alpha}$ within the Ovsynch/TAI protocol varied from 0 to 24% among several studies. The reason for this variation is not clear. A possible explanation is related to the stage of the cycle at initiation of the Ovsynch/TAI protocol.

In a limited data set, Moreira et al. (2000b) observed that CL regression failure only occurred among heifers that initiated the Ovsynch/TAI protocol during the proestrus phase (2/5). In these two heifers, the first injection of GnRH induced the formation of a new CL. This CL showed signs of regression after $PGF_{2\alpha}$, but development was reinitiated after the second injection of GnRH, as determined by ultrasonography and by plasma P_4 concentrations collected daily after the $PGF_{2\alpha}$ injection the Ovsynch/TAI protocol. Subsequently, both accessory CL induced by the first and the second injection of GnRH in both heifers regressed spontaneously approximately 17 d after the CL induced by the first injection of GnRH initiated their development.

At that time, it was hypothesized that cows that initiate the Ovsynch/TAI protocol during proestrus have already experienced regression of the original CL and, most probably, would ovulate to an injection of GnRH. Because an injection of GnRH induces ovulation within approximately 28 h (Pursley et al., 1995), a new CL would be induced 2 d later. Such a CL would be only 5 to 6 d old when $PGF_{2\alpha}$ is administered 7 d after the first GnRH injection and, therefore, would be at the borderline of being unresponsive to an injection of $PGF_{2\alpha}$ (Watts and Fuquay, 1985; Twagiramungu et al., 1995).

If such a model is correct, it would be expected that cows classified as LOW-HIGH, according to plasma P_4 concentrations at the first injection of GnRH and at the injection of $PGF_{2\alpha}$ 7 d later, would be at greater risk for failure of complete CL regression. Such an effect was not observed in experiments conducted by Moreira et al. (1998) because the frequency of cows having incomplete CL regression was not greater among LOW-HIGH cows. Moreover, Vasconcelos et al. (1999b) observed that 7.2% (9/125) of cows submitted to the Ovsynch/TAI protocol failed to undergo complete CL regression 48 h after $PGF_{2\alpha}$ even though all such cows initiated the Ovsynch/TAI protocol between

Days 1 and 9 of the estrous cycle. Thus, if it is true that initiation of the Ovsynch/TAI protocol during proestrus may lead to incomplete CL regression, such an effect does not account for all cases of CL regression failure after $PGF_{2\alpha}$ administration.

Another possible explanation may be related to the composition of the diets fed in different experiments. Concentrations of certain specific fatty acids within a ration may diminish the release of endogenous $PGF_{2\alpha}$ from the uterus (Mattos et al., 2000) and may interfere with the capacity of exogenous $PGF_{2\alpha}$ to completely regress the CL. Furthermore, analyses from Moreira et al. (1998) indicated that frequency of CL regression failure was greater among multiparous cows when compared to primiparous cows. Such an observation may indicate that among primiparous cows, which are smaller in size and have smaller lung capacity to metabolize $PGF_{2\alpha}$, the standard 25 mg dose of $PGF_{2\alpha}$ is enough to induce complete CL regression but that may not be true among multiparous cows. In their initial experiment, Pursley et al. (1995) used a 35 mg dose of $PGF_{2\alpha}$ to induce luteolysis and obtained complete CL regression among all cows. Nonetheless, in subsequent experiments where the incidence of incomplete CL regression was low

(approximately 5.0%, as described above), the dose used for $PGF_{2\alpha}$ was 25 mg.

It is important to note that, although the phenomenon of incomplete CL regression is not clearly understood, most of the data presented above indicates that pregnancy rates for such a group of cows are substantially reduced. One reason for such an effect is related to the fact that, under an elevated P_4 environment, final development of the pre-ovulatory follicle and of the oocyte may be compromised. The pulsatile LH release between luteolysis and the ovulatory LH surge is essential for maturation of the preovulatory follicle (Rahe et al., 1980) and for maturation of the oocyte and resumption of the ribosomal transcription in cattle oocytes (Greeve et al., 1995). If plasma P_4 concentrations are elevated during the final maturation process, the pulsatile release of LH may be reduced and, thus, final follicular and oocyte may be impaired. Although incomplete CL regression may not be the sole reason for short inter-insemination intervals (Schmitt et al., 1996), failure of complete CL regression after $PGF_{2\alpha}$ injection was determined to induce heifers to return to estrus less than 16 d after insemination (Moreira et al., 2000b). Even if a conception to a timed insemination occurs, the chances for embryonic survival are reduced if a

luteolytic signal coming from the uterine endometrium takes place less than 16 d after insemination. Thus, further research is necessary to improve our understanding of the causes for incomplete CL regression after the administration of $PGF_{2\alpha}$ within the Ovsynch/TAI protocol.

Collectively, the evidence provided above leads to the conclusion that stage of the estrous cycle at which the Ovsynch/TAI protocol is initiated affects subsequent pregnancy rates to the synchronized service. Interpretation of the data compiled above indicates that the optimal stage for initiation of the Ovsynch/TAI protocol corresponds to the early diestrous stage (i.e., between d 5 and d 12 of the cycle). Vasconcelos et al. (1999b) reported that pregnancy rates were increased in cows that had initiated the program between d 5 to 13 of the cycle (36.0%, 68/189) compared to cows that received the first GnRH injection at other stages of the cycle (25.0%, 54/215). In addition, when cows initiated the Ovsynch/TAI protocol 7 d after estrus, pregnancy rates were higher compared to conception rates of cows initiating the Ovsynch/TAI protocol at random stages of the cycle (Keister et al., 1999). Thus, data from field experiments support the hypothesis that pregnancy rates are increased when the

Ovsynch/TAI protocol is initiated during the early diestrous stage of the estrous cycle.

Adjusting the Ovsynch/TAI Protocol

Inefficiencies inherent to the Ovsynch/TAI protocol may lead to synchronization failure of follicular and CL development and reduce subsequent pregnancy rates. If such inefficiencies are corrected, conception rates to the Ovsynch/TAI protocol may achieve the same levels observed when cows are inseminated to a detected estrus, and significantly increase pregnancy rates to the synchronized service. Interpretation of the data available at this point indicates that conception to the Ovsynch/TAI protocol may be increased by two methods: use of exogenous progesterone or progestins during the Ovsynch/TAI protocol and pre-synchronization of animals to target initiation of the synchronization program at stages of the estrous cycle considered optimal.

One of the most evident problems is the occurrence of estrus prior to the second injection of GnRH of the Ovsynch/TAI protocol. Premature occurrence of estrus may lead to an asynchrony of ovulation relative to the timed insemination (Moreira et al., 2000b). This way, because ovulation occurs prior to the timed insemination, the

chances for a successful conception are greatly reduced. If cows are detected in estrus and inseminated prior to the second injection of GnRH of the Ovsynch/TAI protocol, conception rates to such service are similar to conception rates of cows bred at observed estrus (Schmidt et al., 1996; Stevenson et al., 1999). But such a practice defeats the purpose of a timed insemination protocol, which is elimination of the labor and inefficiencies associated with estrous detection. On the other hand, correction of the problem of asynchrony of ovulation should result in increased conception rates and still eliminate the need for estrous detection. One possible solution is the use of exogenous progesterone or progestins within the Ovsynch/TAI protocol.

Asynchrony of ovulation occurs due to premature CL regression, which takes place prior to the injection of $PGF_{2\alpha}$ (Moreira et al., 2000b). The premature demise of the CL, which decreases plasma P_4 concentrations, allows for the final development of an estrogenic pre-ovulatory follicle. The increased production of estradiol by the follicle, coupled with low plasma P_4 concentrations, is responsible for an increase in LH pulsatility that culminates with a LH surge and induces ovulation (Caraty et al., 1995; Kesner et al., 1981). However, administration of exogenous

progestins blocks the induction of the LH surge and, therefore, does not allow the ovulation of the follicle (Savio et al., 1993). In this manner, ovulation of the dominant follicle may be controlled and only allowed to occur after the exogenous progestins source is removed.

There are several different progesterone and progestin-delivery devices that have been tested such as Synchro-Mate-B implants (ear implants containing norgestomet), CIDR (an intravaginal device that delivers progesterone), melengesterol acetate (MGA; an oral progestin), and medroxyacetate progesterone sponges (MAP sponges; a cylindrical sponge which is impregnated with MAP and inserted in the vagina). The use of such or any other progesterone releasing devices and feed additives in lactating dairy cows is currently not allowed in the United States, and this fact limits their practical use. Nonetheless, several recent studies have been performed to test the use of such compounds to enhance pregnancy rates to the Ovsynch/TAI protocol.

A series of abstracts observed the effect of adding exogenous progesterone to the Ovsynch/TAI protocol were published at the 2000 American Dairy Science Association and American Society of Animal Science Joint Annual meeting. El-Zarkouny et al. (2000) reported that insertion

of a CIDR device on the day of first GnRH injection and removal at the day of $PGF_{2\alpha}$ injection increased pregnancy rates to a timed insemination when compared to the standard Ovsynh/TAI protocol (39% > 21%). However, authors also noted that over 50% of experimental cows were not cyclic at the time of insemination. Lamb et al. (2000) reported a trend (P = 0.13) for increased pregnancy rates when a CIDR device was added to a Cosynch protocol (i.e., cows receive the second GnRH injection and are timed inseminated 48 h after the injection of $PGF_{2\alpha}$. It was also reported that body condition and days postpartum affected the overall efficiency of the timed insemination protocol. In contrast, Johnson et al. (2000) failed to observe any effects of adding a CIDR to the Ovsynch/TAI protocol. Cows averaged 71 d postpartum at initiation of the experiment, which might indicate that the frequency of anestrous cows was not as high as previously observed in the experiment by El-Zakourny et al. (2000). Such a discrepancy among results from different experiments may be related to the frequency of anestrous cows.

Supplementation with additional progesterone stimulates cows to return to a cyclic status (Anderson et al., 1996; Favero et al., 1995; Fike et al., 1997 Miksch et al., 1978; Smith et al., 1987; Yavas et al., 1999). Therefore, in

cases where the frequency of anestrous cows is high, as described by El-Zakourny et al. (2000), the exogenous progesterone may increase subsequent pregnancy rates. However, in situations where most cows are cyclic, the addition of exogenous progesterone may not have an effect on pregnancy rates.

In support of such a speculation, data from Thatcher et al. (2001) indicated that pregnancy rates of heifers that initiated the Ovsynch/TAI protocol at late diestrus were not affected by supplementation of cows with MGA for 5 or 6 d. Authors speculated that the decrease in pregnancy rates of heifers that initiated the protocol at late diestrus might be related to the ovulation of an aged oocyte in heifers that regressed their CL by the day of $PGF_{2\alpha}$ injection. Another interpretation is that the source of supplemental progesterone was removed too early to avoid an asynchrony of ovulation. Moreira et al. (2000a) inserted a MAP sponge one day after the first GnRH of the Ovsynch/TAI protocol and removed it at the time of $PGF_{2\alpha}$ injection or 12 h later in cows that initiated the protocol at late diestrus (i.e., d 15 of the cycle). Although the addition of the MAP sponge appeared to have alleviated the problem of asynchrony, 13.3% of cows (2/15) ovulated prior to the time of insemination.

If the source of exogenous progesterone needs to be maintained for a longer period (i.e., 24 h after injection of $PGF_{2\alpha}$) to eliminate asynchrony of ovulation, the quality of the follicle may be affected in cows that regress their CL prior to injection of $PGF_{2\alpha}$. Nonetheless, data from Ryan et al. (1995) indicated that the decrease in conception rates observed after a treatment with CIDR for 8 d and an injection of $PGF_{2\alpha}$ on d 7 was eliminated if cows were treated with GnRH or estradiol at d 0. These results indicate that normal fertility could be maintained if a follicle recruited by GnRH or estradiol develops under a low progesterone environment provided by a CIDR for 8 d. In contrast, Xu et al. (2000) and Xu and Burton (2000) observed that conception rates in a Select Synch protocol (i.e., GnRH followed 7 d later by $PGF_{2\alpha}$ and cows are inseminated at detected estrus) were reduced if a CIDR was maintained for 8 d as compared to 7 d. However, treatment groups for the previous comparison was not contemporaneous, and, therefore, these results must be interpreted with care.

The second alternative to alleviate problems associated with the Ovsynch/TAI protocol is pre-synchronization. Most of the problems described above (i.e., failure of

follicular synchronization, premature CL regression) are related to the stage of the estrous cycle at initiation of the Ovsynch/TAI. It follows that, if initiation of the Ovsynch/TAI protocol is targeted to stages of the cycle considered optimal, there is an opportunity to increase the efficacy of the synchronization program. Data presented above indicates that the optimal stage of the cycle for initiation of the Ovsynch/TAI protocol is the early diestrus phase of the estrous cycle (Moreira et al., 2000b; Vasconcelos et al., 1999b). Cordoba and Fricke (2000) injected lactating cows with $PGF_{2\alpha}$ 12 d before the initiation of the Ovsynch/TAI protocol and did not observe any differences in subsequent pregnancy rates as compared to when the Ovsynch/TAI protocol was initiated at random stages of the cycle. It also was observed that approximately 27% of cows were in anestrus at initiation of the previous experiment. In contrast, Cartmill et al. (2000) observed that an injection of $PGF_{2\alpha}$ 12 d prior to initiation of the Ovsynch/TAI protocol increased pregnancy rates as compared to the standard Ovsynch/TAI program. In the previous experiment, authors indicated that the percentage of cows in the early luteal phase (i.e., Days 5 to 9 of the cycle) was increased if $PGF_{2\alpha}$ was injected 12 d

prior to initiation of the timed insemination protocol. Interpretation of these results indicates that a single $PGF_{2\alpha}$ injection prior to the Ovsynch/TAI protocol can increase subsequent pregnancy rates. Nonetheless, such an increase in pregnancy rates may be affected by the frequency of anestrous cows within the herd.

Effects of Body Condition and Anestrus on Fertility to the Ovsynch/TAI Protocol

Body condition score (BCS) is a subjective estimate of the amount of subcutaneous fat on a cow (Edmonson et al., 1989). The most common scoring system for dairy cattle classifies cows from 1 (emaciated) to 5 (obese) with different decimal increases (Edmonson et al., 1989; Fergunson et al., 1992; Wildman et al., 1982).

A relationship between BCS and reproductive performance has been described. Cows with low BCS at parturition have poor reproductive performance (Markusfeld et al., 1997; Suriyasathaporn et al., 1988). Moreover, cows that lose more than 1 unit in BCS after parturition have reduced reproductive performance (Butler and Smith, 1989; Domecq et al., 1997; Nebel and McGillirad, 1993; Ruegg et al., 1992, Ruegg and Milton, 1995; Suriyasathaporn et al., 1988). Nonetheless, a relationship between BCS at breeding and conception rates was not observed

(Garnsworthy, 1988). Failure to observe effects of BCS in the previous study may have been a consequence of confounding factors such as stage of lactation at the time of breeding.

Stevenson et al. (1996) observed that conception rates were greater for cows with higher BCS (> 2.5; 57.4%, 31/54) than for cows with poor BCS (< 2.5; 20.8%, 5/24) at initiation of different estrous synchronization treatments. Burke et al. (1996) reported that BCS at the initiation of the Ovsynch/TAI protocol or a control group (GnRH followed 7 d later with $PGF_{2\alpha}$ and cows inseminated at detected estrus) was positively associated with first-service pregnancy rates. A 13% increase in pregnancy rates was associated with every unit increase in BCS. Similarly, in a study comparing the Ovsynch/TAI protocol and different control groups, Stevenson et al. (1999) observed that for every unit increase in BCS, conception rates increased by 8.6% in experiment 1 and by 10.0% in experiment 2. Moreira et al. (2000c) compared pregnancy rates to the Ovsynch/TAI protocol between cows with poor BCS (≤ 2.5) and a control group where cows had a BCS > 2.5. Pregnancy rates to the Ovsynch/TAI protocol were greater for the control group than for the low BCS group (25.6% > 11.1%). Authors concluded that body condition should be considered when

determining the time postpartum that cows are to be inseminated.

The effect of BCS at initiation of the Ovsynch/TAI protocol is probably associated with the incidence of cows in anestrus. Cows with greater loss in BCS during early lactation have delayed return to cyclicity (Markusfeld et al., 1997). Moreover, it was observed that cows with low plasma progesterone concentrations at first GnRH and at $PGF_{2\alpha}$ (i.e., LOW-LOW cows) had lower pregnancy rates to the Ovsynch/TAI protocol (Moreira et al., 2000c; Moreira et al., 2000d; Stevenson et al., 1999).

Ovsynch/TAI During Summer Heat Stress

Wilson et al. (1998b) hypothesized that lactating dairy cows are more susceptible to heat stress during summer because of elevated internal heat production associated with lactation. Due to heat stress, reproductive performance of cattle is compromised (Gwazdauskas, 1985). Pregnancy rates decline as a result of decreased conception rates (Badinga et al., 1985; Thatcher and Collier, 1986) and reduced estrous detection rates (Thatcher and Collier, 1986; Her et al., 1988). Conception rates are decreased because elevated body temperatures results in early embryonic death (Putney et

al., 1988). Estrous detection rates are affected because of altered dominant follicle development and function (Badinga et al., 1993), which leads to reduced plasma concentrations of estradiol during proestrus (Gwazdauskas et al., 1981). Although a timed insemination system may not relieve the problem associated with high embryonic losses, the use of the Ovsynch/TAI protocol in heat stressed cows may increase pregnancy rates by eliminating the need for estrous detection.

Such a hypothesis was tested in two experiments in Florida. Aréchiga et al. (1998) observed an increase in pregnancy rates for the Ovsynch/TAI group compared to a control group, which was inseminated at detected estrus. Cumulative pregnancy rates from first service to 120 d postpartum also were increased in the Ovsynch/TAI group in the previous experiment. De la Sota et al. (1998) observed that, although conception rates were greater for the control group (i.e., insemination at detected estrus after a single injection of $PGF_{2\alpha}$), first-service pregnancy rates were greater for the Ovsynch/TAI group. The increase in pregnancy rates for the Ovsynch/TAI group was associated with a sizable increase in submission rates (or estrous detection rates): 18.1% for the control group as compared to 100% for the Ovsynch/TAI group. Moreover, pregnancy

rates at 120 d postpartum were increased and the number of days open at 120 d postpartum was decreased for cows in the Ovsynch/TAI group compared to the control group. Thus, because it eliminates the need for estrous detection and increases submission rates, the Ovsynch/TAI protocol may be an important tool in the reproductive management of heat-stressed cattle.

Bovine Somatotropin and Reproduction

It has been well documented that administration of bovine somatotropin (bST) increases milk production of lactating dairy cows. This effect was described originally by Azimov and Krouze (1937). In 1957, Hutton demonstrated that bST was particularly effective after peak lactation. After the advent of recombinant DNA technology, it became feasible to produce bST in an industrial scale. Since its approval for sale by the FDA in November 1993, bST has rapidly become the largest selling drug for dairy cattle in the US. Current estimates by the Monsanto Co., which sells the only commercially available bST product, Posilac, indicates that approximately one third of the dairy cows in the United States are in herds that are currently using bST.

Daily injections of highly purified somatotropin increased milk yield by 10 to 45% (Peel and Bauman, 1987). Increases in milk production with the use of Posilac, the commercially available sustained-release formulation of bST, ranged from 11 to 31% (Pell et al., 1992; Remond et al., 1991). The effects of bST on milk production are modulated via an increase in the production of insulin-like growth factor-I (IGF-I) by the liver (Peel and Bauman, 1987). However, initial studies have suggested that the use of bST was associated with a decrease in reproductive performance of lactating cows. Such a decrease was probably caused by a combination of direct effects of bST and indirect effects via IGF-I (Kirby et al., 1997a).

Effects of bST on Fertility of Dairy Cows

Most of the reviewed experiments suggested that treatment of lactating dairy cows with bST decreased reproductive performance. A summary of the results from these experiments is depicted on Table 2.2. A common observation among these experiments is the fact that bST-treated cows had a slower rate of body weight gain (Burton et al., 1990) or a decrease in body condition scores (Morbeck et al., 1991). Such an observation undoubtedly is associated with increased energy demands that must

accompany the increased milk production of bST-treated cows. Therefore, the decreased reproductive performance of cows receiving bST was associated with an increased depth or length of negative energy balance after parturition.

A prolonged period of negative energy balance may result in a prolonged period of anestrus and increased interval from parturition to resumption of ovarian activity and conception, as well as to a decreased rate of estrous detection (Canfield and Butler, 1990). Furthermore, negative energy balance decreases weight of the CL in heifers (Villa-Godoy et al., 1990; VandeHaar et al., 1995) and the concentration of progesterone in blood and milk of lactating dairy cows (Villa-Godoy et al., 1988; Spicer et al., 1990). Because progesterone is necessary for maintenance of pregnancy, it is possible that the effect of negative energy balance on the ovary may decrease fertility of lactating cows (Yung et al., 1996). Prolonged negative energy balance during the postpartum period also decreases the dominance period and the maximum diameter of dominant follicles (Burns et al., 1994) probably due to decreased LH pulsatility (Canfield and Butler, 1990) and this may constitute a reason for reduced reproductive performance in lactating dairy cows.

Table 2.2. Effects of various doses of bST treatment initiated at different days postpartum (d PP) on reproductive performance of lactating dairy cows.

Reference	Dose	d PP	Results
Burton et al., 1990	10.3 - 41.2 mg/d	28-35	. Greater days open
Morbeck et al., 1991	5.15 - 16.5 mg/d	28-35	. Lower estrous detection . Greater interval from parturition to first AI
Hemken et al., 1991	20.6 mg/d	28-35	. Lower conception and pregnancy rates . Greater interval from first estrus to first AI
Zhao et al., 1992	10.3 mg/d or 350 mg/14 d	28-35	. Increased days to first AI
Cole et al., 1992	0.6 - 3.0 mg/14 d	60	. Lower conception rates . Greater interval from first estrus to AI
Hansen et al., 1994	5.15 - 16.5 mg/d	28-35	. Increased days to conception . Lower conception rates
Esteban et al., 1994	17.2 - 86.0 mg/d	70	. Increased behavioral anestrus . Lower pregnancy rates
Moallem et al., 1997	500 mg/14 d	10	. Greater interval from parturition to estrus
Luna-Dominguez et al., 2000	500 mg/14 d	60	. Lower pregnancy rates

Another common factor among the above-described studies is that bST administration was initiated prior to or at the fifth week of lactation (with the exception of Cole et al., 1992, Esteban et al., 1994, and Luna-Dominguez et al., 2000). In other experiments, where bST treatment was initiated after the ninth week of lactation, bST had no effect on the reproductive performance of lactating cows (Erdman et al., 1990; Judge et al., 1999; Nytes et al., 1990; Oldenbroek and Garssen, 1993; Pell et al., 1992;

Remond et al., 1991). Collectively, these observations indicate that the deleterious effects of bST on reproductive performance of lactating cows are either attenuated or eliminated if treatment is initiated only after the ninth week of lactation. Because most lactating cows may be recovering from the negative energy balance effects after the ninth week of lactation, it is possible that initiation of bST treatment at this stage of lactation does not affect significantly the energy balance of cows and lead to decreased reproductive performance. Indeed, in most studies using the commercial available formulation that contains 500 mg of bST injected biweekly, where treatment was initiated after the ninth week of lactation (according to label instructions), no deleterious effects on reproductive performance of lactating cows were detected (Pell et al., 1992; Remond et al., 1991; Judge et al., 1999). Only one study reported a decrease in fertility among bST-treated cows (Luna-Dominguez et al., 2000). Furthermore, no differences in the percentage of cows being culled due to reproductive reasons were observed among cows receiving bST treatment (Ruegg et al., 1998).

In addition to temporary negative energy balance, another possible explanation for the decreased reproductive efficiency is a possible inhibition of behavioral estrus in

cows treated with bST. Morbeck et al. (1991) and Waterman et al. (1993) had indicated a decreased rate of estrous detection among lactating cows receiving bST treatment. Cole et al. (1992) observed an increase in the interval from first estrus to first insemination and attributed such an effect to reduced expression of estrous behavior among bST-treated cows. This reduction in estrous expression was associated with an increased negative energy balance in cows receiving bST. However, Lefebvre and Block (1992) observed that estrous expression was reduced after bST treatment in ovariectomized and steroid-primed heifers. Authors concluded that bST altered behavioral centers within the brain that control estrous expression. Similarly, transgenic mice that express a bST transgene or normal mice administered with bST also showed decreased intensity of mating behavior (Cecim et al., 1995). Kirby et al. (1997b) detected an inhibitory effect of bST on the expression of estrus and an increase in the percentage of undetected ovulations. Such a decrease in behavioral estrus was not associated with differences in steroid concentrations because both progesterone and estradiol concentrations were not affected by bST treatment. Thus, other direct effects on behavioral centers within the brain

are more likely to be responsible for the decreased estrous expression in bST-treated cows (Kirby et al., 1997b).

Interestingly, Cole et al. (1992) reported an increased in twinning rates among cows treated with bST. Such an increase in twinning rates was not replicated in subsequent studies (Collier et al., 1997). The failure to observe a consistent effect of bST on twinning in dairy cattle may reflect an interaction of bST with either genetic or environmental factors (Lucy, 2000). Moreover, as will be discussed later, bST treatment did not affect ovulation rates in dairy cows and increases in twinning rates may be associated with an increased embryo survival in bST-treated cows (Kirby et al., 1997a). Also, there was some concern that the use of bST and subsequent increased IGF-I concentrations may lead to an alteration in calf birth weights and, perhaps, to a "large offspring syndrome". Results from two experiments, however, did not indicate any increase of calf birth weight effect due to bST treatment (Gallo and Block, 1990; Oldenbroek et al., 1993).

Interestingly, when estrous detection was eliminated through the use of a timed insemination protocol, not only was the deleterious effect of bST on fertility not observed, but there was an increase in first-service

pregnancy rates when cows received bST treatment (Moreira et al., 2000d). Similar results were observed in other studies. Bilby et al. (1999) treated dairy and beef cows with a single 167 mg dose of the sustained-release formula of bST commercially available for producers (i.e., one third of the indicated dose) at insemination. Results indicated a numerical increase (+ 4.9%) in pregnancy rates of cows treated with low doses of bST, but results varied according to location and the increase in pregnancy rates was not statistically significant. In a more recent study, Hernandez-Ceron et al., (2000) also reported increased pregnancy rates in cows having more than one service that were treated with a single full dose of bST (500 mg of the sustained-release formula). Thus, in situations where estrous detection was eliminated or where treatment was administered after estrous detection, use of bST improved reproductive performance of cows. The study of the effects of bST on the reproductive system of cows may provide important insights as to the mechanisms by which bST, either directly or via the IGF-I system, may increase pregnancy rates of cows. Accordingly, the remainder of this literature review will focus on the effects of bST and IGF-I on the ovary, oviduct/uterus and preimplantation embryo.

Effects of bST on CL Development

Receptors for bST (or growth hormone; GH) were detected in the bovine CL (Lucy et al., 1993b; Lucy et al., 1995a) and the CL contained more GH receptor mRNA than other reproductive tissues examined such as the ovary without luteal tissue, oviduct, and endometrium (Kirby et al., 1996). Moreover, receptors for IGF-I are expressed in the bovine CL (Spicer and Echternkamp, 1995). Chase et al. (1998) studied CL development in cattle with growth hormone receptor deficiency and observed that this type of cattle has decreased CL size and lower plasma progesterone compared to normal cows. Authors concluded that GH and/or IGF-I are required for normal CL formation and function.

The effects of bST treatment on CL formation and development are not clear though. While some studies observed no effects of bST treatment on the cross-sectional area of the CL or plasma progesterone concentrations during the estrous cycle (Dalton and Marcinkowski, 1994; De la Sota et al., 1993; Gong et al., 1993a; Kirby et al., 1997b; Lucy et al., 1993a; Lucy et al., 1994a), others found increased CL weight and plasma progesterone concentrations in bST-treated cows (Gallo and Block, 1991; Lucy et al., 1994b; Lucy et al., 1995b; Schemm et al., 1990). In

addition, a stimulatory effect of GH on progesterone synthesis was demonstrated in luteinized granulosa cells (Wathes et al., 1995). Microdialysed bovine CL perfused with different concentrations of GH showed a dose-dependent stimulation of progesterone release especially in CL collected during early stages of the estrous cycle (Days 5 to 7) and early pregnancy (Days 60 to 120; Lieberman and Schams, 1994).

In vivo, the ruminant CL produces low quantities of IGF-I (Kirby et al., 1996), and luteal IGF-I synthesis was not changed by negative energy balance (VandeHaar et al., 1995), administration of bST (Kirby et al., 1996), or blocking of endogenous GH by immunization against GH-releasing factor (Cohick et al., 1996). However, addition of IGF-I increased progesterone synthesis in cultured luteal cells (Constantino et al., 1991; McArdle and Holtorf, 1989; Sauerwein et al., 1992). Collectively, these data suggests that both GH and IGF-I contribute to CL formation and development, but such an effect is either not a strong one or may be modulated by other factors, such as energy balance. Administration of bST increased IGF-I concentrations but failed to improve CL development in heifers in negative energy balance (Yung et al., 1996). Jimenez-Krassel et al., (1999) indicated that negative

energy balance, lactation status, timing, duration of bST treatment and type of bST injections (i.e., infusion, daily, biweekly) could explain the discrepancies.

If bST influences CL formation, development and function, several hypotheses may be suggested. Administration of bST may increase the number of granulosa and theca cells of the pre-ovulatory follicle and result in a greater number of luteal cells after ovulation. Nonetheless, as it will be further discussed, bST administration did not affect the growth and size of the preovulatory follicle (De la Sota et al., 1993; Kirby et al., 1997a). Alternatively, it is possible that differentiation of granulosa and theca cells into luteal cells may have been stimulated by bST. Treatment of cows with bST increased intra-follicular concentrations of GH (Jimenez-Krassel et al., 1999) and IGF-I (Lucy et al., 1995b) and both GH and IGF-I receptors were detected in bovine ovarian follicles (Izadyar et al., 1997; Spicer and Echternkamp, 1995). Therefore, such an alternative hypothesis seems reasonable, but bST treatment failed to affect CL development when administered during the peri-ovulatory period (Lucy et al., 1994a). Moreover, a direct effect of GH on the steroidogenic capacity of large luteal cells (where GH receptors are present; Lucy et al., 1993b)

or an increased differentiation of small into large luteal cells (Donaldson and Hansel, 1965) was suggested (Lucy et al., 1995b).

It has been proposed that GH induces steroidogenesis directly or by potentiating gonadotropin action (Hull and Harvey, 2000). Authors hypothesized that GH may up-regulate LH receptors, thus enhancing LH-induced luteinization and the acquisition of progesterone synthetic ability. This possibility is supported by the inability of GH to induce progesterone production in the absence of gonadotropins in rats (Jia et al., 1986). In the bovine, results are ambiguous though. Addition of GH stimulated the production of progesterone in microdialysed bovine CL (Lieberman and Schams, 1994), suggesting that bST may act directly and independently from gonadotropins, but such an effect of bST was not observed when luteal cells were used in an in vitro model (Hansel and Seifart, 1968). Moreover, IGF-I has been shown to stimulate steroidogenesis in cultured bovine CL (Einspanier et al., 1990; Sauerwein et al., 1992). Increased IGF-I may also have lead to increased DNA synthesis (Chakravorty et al., 1993) and growth of the CL. Therefore, both bST and IGF-I may be acting to stimulate CL development.

Effect of bST on Follicular Development

Growth hormone receptor mRNA and protein were detected in bovine ovarian follicles, granulosa cells, cumulus cells and oocytes (Izadyar et al., 1997; Kolle et al., 1998; Lucy et al., 1993b). In addition, GH mRNA and/or GH protein were identified in granulosa and theca cells, as well on cells of the cumulus and oocyte (Izadyar et al., 1999). Components of the IGF system (IGF-I, IGF-I receptors and IGF-I binding proteins) are expressed within granulosa and theca cells (Armstrong and Webb, 1997; Giudice, 1992; Spicer and Echternkamp, 1995). Ovarian follicles are able to synthesize IGF-I (Giudice, 1992; Hammond et al., 1991; Perks et al., 1995; Spicer and Echternkamp, 1995), and the synergistic actions of IGF-I, LH, and FSH are involved in follicular growth (Hammond et al., 1991). Collectively, the observation that receptors for both GH and IGF-I are present within ovarian follicles as well as the fact that ovarian follicles secrete both GH and IGF-I suggest that both factors may play an important role in follicular development.

Several publications have reported that bST affects follicular development in cows. The most common observation is related to the number of small and medium-sized follicles. Administration of bST increased the

number of Class I and II follicles (De la Sota et al., 1993; Gong et al., 1991; Gong et al., 1993a; Gong et al., 1997; Kirby et al., 1997a) and the increased number of recruited follicles can be maintained for at least 84 d and may persist for 21 d after bST treatment (Kirby et al., 1997a). Lucy et al. (1995b) also observed that bST increased the number of follicles in the 10 to 15 mm size upon administration of bST. Further evidence that bST treatment increases recruitment of small and medium-sized follicles was observed during superovulation of cows. Treatment with bST increased the number of follicles ovulating after superovulation (Gong et al., 1993b; Gong et al., 1996; Rieger et al., 1991). This increase was attributed to an increase in the population of small antral follicles induced by administration of bST (Gong et al., 1993b; Gong et al., 1996).

Different hypotheses have been suggested to explain the above-mentioned effects. In mice, treatment with somatotropin increased pre-antral follicular growth in vitro (Liu et al., 1998). Hence, bST may be stimulating the number of follicles that leave the pool of pre-antral follicles during the recruitment phase. However, the level of somatotropin receptor expression in bovine antral follicles is relatively low (Lucy et al., 1993b; Yuan and

Lucy, 1996) and, therefore, the functional significance of bST for antral follicular growth remains to be established (Lucy, 2000).

Several lines of evidence support the possibility that increased IGF-I in blood after bST treatment mediate the effects of bST. Heifers treated with increasing doses of bST failed to have greater growth of antral follicles when the bST dose was below the threshold for increased IGF-I (Gong et al., 1997). Miniature cattle with high blood somatotropin but low IGF-I concentrations had one-third the number of small antral follicles compared with control cattle in the same herd (Chase et al., 1998). Moreover, based on in vitro data, the most likely mechanism for increased follicular growth is the synergistic effect of IGF-I on expression or action of gonadotropin receptors (Lucy, 2000). In vivo data does not support such a hypothesis in cattle because bST treatment did not increase gonadotropin-binding sites (Gong et al., 1991; Andrade et al., 1996). The possibility that bST or IGF-I increases the in vivo activity of gonadotropin second messenger pathways in cattle has not been addressed (Lucy, 2000).

An additional possibility is that bST treatment may decrease atresia among the pool of recruited follicles. In cattle, bST treatment decreased follicular atresia (Cushman

et al., 1996). In sheep, GH treatment did not affect the number of follicles between 0.5 and 0.8 mm nor the mitotic index of follicles larger than 0.8 mm but significantly reduced the number of early atretic follicles in the 0.8 to 2.0 mm category (Joyce et al., 2000). Because GH treatment did not affect FSH or LH concentrations, it was concluded that the anti-atretic effect of GH was not mediated through changes in the gonadotropin secretion pattern. Furthermore, it was suggested that increased IGF-I concentration after GH treatment were responsible for the anti-apoptotic effects observed in sheep (Joyce et al., 2000) because IGF-I mediates the anti-apoptotic effect of GH and FSH in rat whole-follicle culture (Chun et al., 1994; Eisenhauer et al., 1995).

Despite the observed effect of bST on small and medium-sized follicles, there was no effect of bST on growth and size of the dominant follicle in several studies (De la Sota, 1993; Gong et al., 1993; Kirby et al., 1997a; Lucy et al., 1995b) or an effect on the dominance mechanisms exerted by the dominant follicle (Gong et al., 1993). However, the timing of emergence of follicular waves was altered in bST-treated cattle. The duration of the dominant phase of the first wave dominant follicle was shortened by about 2 d when bST was administered (Kirby et

al., 1997a; Lucy et al., 1994b). This led to an earlier emergence of the second wave dominant follicle and such a shift was associated with a correspondent shift in the timing of FSH peak (Kirby et al., 1997a). Hence, although bST treatment did not affect the rate of growth, size, or the dominance mechanisms of the dominant follicle, the length of the dominance phase was shortened.

The reduction in the length of the dominance phase of dominant follicles was associated with changes in intra-follicular concentrations of IGF-I and its binding proteins (IGFBP). Under control conditions, IGFBP are decreased in the follicular fluid of dominant follicles, and IGF availability is presumably increased (Lucy, 2000) and the concentrations of IGFBP are increased in atretic follicles (Besnard et al., 1996; De la Sota et al., 1996; Stewart et al., 1996). Follicular fluid of dominant follicles from bST-treated cattle had increased amounts of low-molecular weight IGFBP (Jimenez-Krassel et al., 1999; Lucy et al., 1995b). Therefore, the premature loss of dominance in cows receiving bST may be a result of the accumulation of IGFBP in follicular fluid (Lucy, 2000).

Alternatively, bST may affect the dominant follicle through its effects on energy balance. The use of bST was associated with a transient negative energy balance

(Etherton and Bauman, 1998) and negative energy balance may lead to a decreased LH pulsatility (Canfield and Butler, 1990). A decrease in LH pulsatility could lead to a premature loss of dominance and greater development of subordinate follicles (Lucy, 2000). Nonetheless, effects of bST on follicular growth and dominance also were detected in nonlactating heifers (Gong et al., 1991; Gong et al., 1993; Gong et al., 1997; Lucy et al., 1994a). Thus, changes in negative milk production and energy balance may not explain the follicular responses observed (Lucy, 2000).

In conclusion, the effects of bST are primarily exerted on the recruitment phase of follicular growth because selection of large follicles and ovulation rates were unaffected by bST (Kirby et al., 1997a). It follows that, although follicular recruitment is stimulated by bST, only a single follicle is selected and only a single follicle is ovulated (Kirby et al., 1997a). Thus, the reported increase in twinning rates among bST-treated cows (Cole et al., 1992) may not be related to an increase in ovulation rate, but, instead, to an increased likelihood of embryonic survival in cows with double ovulations (Kirby et al., 1997a).

Effects of bST on Oviduct and Uterus

There is evidence that intra-luminal concentration of IGF-I may have an effect on early embryonic development. In pigs, the elongating blastocyst secretes estradiol, which can increase IGF-I secretion by the uterine endometrium into the uterine lumen (Simmen et al., 1990). In sheep, a stimulatory effect of combined IGF-I and IGF-II on the production of ovine interferon-τ was detected suggesting that both IGF-I and IGF-II may stimulate peri-attachment development of the conceptus (Yong et al., 1991). In cattle and sheep, mRNA for both IGF-I and IGF-II were detected in oviductal cells (Schmidt et al., 1994; Stevenson and Wathes, 1996). In ewes, IGF-I mRNA concentrations were greater in both mucosa and muscle layers of the oviduct wall at estrus and were still elevated on d 2 to 3 after ovulation (Stevenson and Wathes, 1996). A similar peak in IGF-I mRNA activity around the ovulatory period has been reported in the cow oviduct (Schmidt et al., 1994). Because ruminant embryos reach the 8- to 16-cell stage in the oviduct (Hunter, 1988) where the embryo is in intimate contact with the epithelial lining of the ampullary mucosa, perhaps luminal IGF-I concentrations play an important role during early embryonic development. Moreover, mRNA for IGF-I was identified throughout the pre-

implantation development of bovine and sheep embryos, from the two-cell stage to the hatched blastocyst (Lonergan et al., 1999; Lonergan et al., 2000; Watson et al., 1992; Watson et al., 1994). Thus the embryo is capable of producing IGF-I, which can act in an autocrine fashion to stimulate its own development.

With these observations on mind, Wathes et al. (1998) proposed two mechanisms by which IGF-I plays a role during early embryonic development: IGF-I may be released into the oviductal fluid to stimulate embryo development directly, or, alternatively (or in addition), IGF-I from both oviduct and embryo may stimulate the secretory activity of the ampulla, which peaks during this period (Murray, 1996). Among such secretions, a number of steroid-modulated glycoproteins are released, which have been postulated to enhance both fertilization rates and the developmental competence of the embryos (Murray, 1996).

As the embryo moves from the oviduct into the uterus, there is a change in the secretory pattern of IGF-I. Concentrations of IGF-I within the uterine lumen are increased between d 0 to 5 of the estrous cycle but only a moderate expression of IGF-I mRNA occurred in the bovine uterus during the luteal phase (Geisert et al., 1991; Kirby et al., 1996). Therefore, maximal concentrations of IGF-I

are present during sperm transport, but several days before the arrival of the embryo (Wathes et al., 1998). Such timing diminishes a potential direct effect of luminal IGF-I on embryonic development after the embryo reaches the uterus, but raises the question of whether luminal concentrations of IGF-I may be important for semen capacitation and transport.

Administration of GH to GH-deficient rats and humans increased semen concentration and motility (Breier et al., 1996; Homburg and Farhi, 1995; Ovesen et al., 1996) and the increase in motility coincided with increased seminal plasma IGF-I concentrations (Homburg and Farhi, 1995; Ovesen et al., 1996). Furthermore, seminal plasma concentrations of IGF-I were positively correlated with sperm count in fertile and infertile men, and it was proposed that seminal plasma concentrations of IGF-I may serve as a marker of semen quality (Colombo and Naz, 1999). In cattle, administration of bST to bulls increased sperm motility and fertility after artificial insemination (Sauerwein et al., 2000) and serum concentrations of IGF-I were correlated with the percentage of normal sperm cells (Yilmaz et al., 1999). Collectively, these observations raise the possibility that IGF-I concentrations in the

uterine lumen of cows may stimulate the fertilization capacity of semen.

Paradoxically, although concentrations of IGF-I within the uterine lumen are decreased during the luteal phase, expression of IGF-I receptors was highest in the mid-luteal phase (Wathes et al., 1998). In the uterus of cow and sheep, IGF-I mRNA is mainly localized in the epithelium of the endometrial glands (Stevenson et al., 1994; Wathes et al., 1998). The high concentration of IGF-I receptors on uterine epithelial glands suggests a role for IGF-I in regulating their secretory activity, which is likely to be important in providing an environment capable of sustaining embryonic development because the pre-implantation embryo depends on such secretions for nutritional support (Wathes et al., 1998).

Collectively, these observations provide evidence that components of the IGF system are physiologically important during the development of pre-implantation embryos. An important question, though, is how bST treatment may affect this interaction between the oviduct/uterus and the developing embryo. The up-regulation of IGF-I mRNA in both oviduct and uterus seems to be driven by the preovulatory increase in circulating concentrations of estradiol (Murphy and Ghahary, 1990). In liver, IGF-I is regulated by GH

(Peel and Bauman, 1987) and GH receptors have been identified within the oviduct and uterus (Kirby et al., 1996; Lucy et al., 1995a). However, concentrations of IGF-I mRNA in the cow uterus and of IGF-I within the uterine lumen were not increased by bST administration (Kirby et al., 1996; Lucy et al., 1995b). Such a lack of bST regulation of IGF-I production by the endometrium was associated with a low level of GH receptors during the end of the luteal phase (Kirby et al., 1996). Nonetheless, both reports collected samples around Days 16 to 17 of the estrous cycle, when IGF-I concentrations are physiologically lower. Further research is necessary to determine whether bST administration can alter the concentrations of IGF-I in both oviduct and uterus at earlier stages of the estrous cycle.

Another level of control of uterine function during early pregnancy in cattle may occur through the actions of IGFBP (Lucy et al., 1995b). Indeed, IGFBP-2 is abundantly expressed within the endometrium of cows (Geisert et al., 1991) suggesting a local role for IGFBP in the uterus. The concentrations of IGFBP-3, -4, and -5 were increased in uterine luminal fluid after cows received bST treatment (Lucy et al., 1995b). Such an increase in the luminal concentrations of IGFBP may be a result of increased gene

expression in the endometrium or increased in blood concentrations of these binding proteins that occur after bST treatment (Lucy et al., 1995b).

Effects of bST on Oocyte and Embryonic Development

There is much experimental evidence that GH has a role in oocyte maturation. Messenger RNA for GH receptors is expressed in the bovine cumulus cells, mural granulosa cells and oocyte (Izadyar et al., 1997b; Kolle et al., 1998). Addition of GH to the maturation medium of bovine oocytes stimulates cumulus expansion and nuclear maturation of oocytes, and increases fertilization rates, as indicated by an increase in cleavage rates (Izadyar et al., 1996; Izadyar et al., 1998a; Izadyar et al., 1998b). Moreover, effects of GH on oocyte maturation in vitro were not mediated through IGF-I because addition of antibody against IGF-I did not inhibit the stimulatory effect of GH (Izadyar et al., 1997b). Initially, data indicated that the effect of GH on oocyte maturation was exerted through cumulus cells, because GH did not improve the maturation of denuded oocytes (Izadyar et al., 1997b). Subsequent experiments indicated that GH also enhanced maturation of denuded oocytes, however (Izadyar et al., 1997a; Izadyar et al., 1998b). This was associated with an improved cytoplasmic

maturation of the oocyte because GH supplementation during maturation enhanced the migration of cortical granules and sperm aster formation (Izadyar et al., 1998a). Cortical granules distribution and sperm aster formation have been used as two reliable indicators for the evaluation of the developmental competence of bovine oocytes (Damiani et al., 1996).

In addition, Izadyar et al. (1997a) observed that the effect of GH on oocyte maturation and cumulus expansion is exerted through the camp signal transduction pathway and not through JAK phosphorylation pathway as previously demonstrated. Furthermore, it was observed that mRNA for GH is expressed and GH protein is produced by granulosa, theca, and cumulus cells and by the immature and mature oocyte (Izadyar et al., 1999), which indicates a possible paracrine and/or autocrine role for GH during oocyte maturation. Collectively, these observations indicate that GH may directly stimulate oocyte maturation and increase fertilization rates. Because GH concentrations within follicular fluid of cows are increased after bST treatment (Jimenez-Krassel et al., 1999), it is possible to speculate that bST administration may stimulate oocyte maturation and enhance fertilization rates.

Addition of GH during oocyte maturation also increased the percentage of oocytes that reached the blastocyst stage after 9 d of culture (Izadyar et al., 1996; Izadyar et al., 1998b). However, such an effect was probably due to the increased cleavage rate. As a percentage of cleaved oocytes, GH did not affect development to the blastocyst stage. Percentage of blastocysts at d 9 for GH treated-oocytes was 34.2% (144/420) as compared to 30.2% (102/338) for controls, as calculated from Izadyar et al. (1996), and 38.1% (107/281) for GH-treated oocytes versus 34.5% (80/232) for control-treated oocytes, as calculated from Izadyar et al. (1998b). Nevertheless, it was recently reported that pre-implantation bovine embryos express mRNA for the GH receptor and that addition of GH to the culture medium increased the development to the blastocyst stage at d 9 (Izadyar et al., 2000). Addition of GH to cultured mouse embryos increased the percentage of blastocysts and the number of blastomeres per blastocyst, but such an effect was eliminated when an anti-GH receptor antibody also was added (Fukaya et al., 1998). Moreover, it was also observed that bovine embryos as early as the 2-cell stage express GH mRNA (Lechniak et al., 1999), which suggests a possible autocrine stimulation of early embryonic development. Therefore, in addition to improving

oocyte maturation, there is strong evidence that GH directly enhances early embryonic development.

Bovine granulosa and cumulus cells have receptors for IGF-I (Spicer et al., 1994; Spicer and Stewart, 1996). Funston et al. (1996) observed an absence of IGFBP other than IGFBP-3 in the follicular fluid of bovine pre-ovulatory follicles in contrast with subordinate follicles and speculated that the absence of IGFBP may allow for increased availability of IGF-I that may be important for oocyte maturation and ovulation. Addition of IGF-I or of IGF-I and epidermal growth factor to the maturation medium of bovine cumulus-oocytes complexes also has been shown to stimulate cumulus expansion and oocyte maturation, and increase cleavage rates (Herrler et al., 1992; Izadyar et al., 1997b; Lorenzo et al., 1994; Rieger et al., 1998; Sakaguchi et al., 2000). Such an effect of IGF-I was also observed in rabbit (Lorenzo et al., 1996), buffalo (Pawshe et al., 1998), and sheep cumulus-oocytes complexes (Guler et al., 2000). However, in contrast with GH, the stimulatory effects of IGF-I on oocyte maturation seem to be exerted through cumulus cells because there was no direct effect of IGF-I on denuded oocytes (Lorenzo et al., 1995; Sakaguchi et al., 2000).

Presence of IGF-I in the culture medium of bovine oocytes also has been shown to stimulate development to the blastocyst stage (Herrler et al., 1992; Kaye et al., 1992; Matsui et al., 1994). Such an effect is possibly a direct one because mRNA for IGF-I receptors is expressed as early as the one-cell stage of development (Watson et al., 1992). Moreover, recent evidence has associated IGF-I production by the early embryo with its developmental capacity. Lonergan et al. (1999) observed that the time interval from insemination to first cleavage has a major influence on subsequent developmental capacity of bovine embryos. Embryos that cleaved 30 h or less after insemination had greater development to the blastocyst stage at d 8 than embryos that only cleaved after 30 h post insemination. In a subsequent study, Lonergan et al. (2000) also demonstrated that IGF-I was synthesized by all embryos that cleaved 27 to 30 h after insemination, whereas IGF-I was only found in some of the embryos that cleaved between 33 and 36 h after insemination and in none of the embryos that cleaved after 36 h post insemination. Thus, authors speculated that IGF-I expression may be linked to the time of first cleavage and may act as an indicator of embryonic developmental capacity. Collectively, these observations support the importance of IGF-I concentrations within the

oviductal lumen and suggest that IGF-I may be acting on a paracrine and/or autocrine fashion to stimulate embryonic development.

Furthermore, it was also reported that addition of IGF-I and IGF-II to the culture medium of bovine oocytes significantly decreased the apoptotic cell index and increased the total cell number of blastocysts at d 7 (Byrne et al., 1999). In humans, such an anti-apoptotic effect of IGF-I also has been demonstrated (Spanos et al., 2000). A combination of IGF-I and IGF-II to culture medium stimulated the secretion of interferon-τ by ovine conceptus (Ko et al., 1991). Based on these observations, it can be hypothesized that in vitro produced embryos cultured with IGF-I may have better chances to survive the freezing process due to a greater number of viable cells and to survive the period of maternal recognition of pregnancy due to increased interferon-τ production.

It also can be hypothesized that bST treatment improves oocyte maturation, increases fertilization rates and improves early embryonic development. However, data from two different experiments failed to observe any positive effects of bST treatment at the time of insemination of superovulated cows on the number or percentage of transferable embryos recovered 7 d later

(Herrler et al., 1994; Kuehner et al., 1993). It must be pointed out though that the number of flushes that were replicated in both of the two previous experiments was relatively low (< 15 flushes per group). In addition, high variability among responses of cows to superovulatory treatments may have reduced the chances of detecting differences between treatments. The effect of bST treatment on the rate of embryonic development was not addressed in the previous studies. Long term treatment of Holstein and beef heifers with bST increased the number of follicles observed at ultrasonography but failed to increase the number of recovered oocytes by consecutive transvaginal aspirations (Bols et al., 1998; Tripp et al., 2000). Also, quality and subsequent development to blastocyst of recovered oocytes were unchanged by treatment (Bols et al., 1998; Tripp et al., 2000). These observations do not support a beneficial effect of bST treatment in vivo. However, Herrler et al., (1994) had observed that concentrations of IGF-I in follicular fluid were only increased in follicles greater than 8 mm in diameter. Furthermore, continuous transvaginal aspirations also may affect the quality of oocytes and induce acyclicity (Bols et al., 1998). Therefore, these observations need to be interpreted with care.

In conclusion, the observations that both bST and IGF-I affect CL function, follicular development, oviduct and uterine function, as well as oocyte maturation and early embryonic development, provide strong support to the observation that bST may increase pregnancy rates of lactating dairy cows submitted to a timed artificial insemination (Moreira et al., 2000d). Among other objectives, this dissertation aims to further study whether the use of bST may indeed increase fertility of lactating cattle.

CHAPTER 3
EFFECTS OF PRE-SYNCHRONIZATION AND BOVINE SOMATOTROPIN ON PREGNANCY RATES TO A TIMED ARTIFICIAL INSEMINATION PROTOCOL IN LACTATING DAIRY COWS

INTRODUCTION

Lactating dairy cows treated with bST were reported to have decreased reproductive performance (Cole et al., 1992; Collier et al., 1997; Zhao et al., 1992). Such an observation could be attributed to effects of bST on estrous expression. Cows treated with bST did not express estrus as intensely as untreated controls, which increased the percentage of undetected ovulations (Kirby et al., 1997b), and steroid-primed, ovariectomized heifers had reduced estrous expression when bST was administered (Lefebvre and Block, 1992). It was hypothesized that bST altered behavioral centers within the brain that control expression of estrus (Lefebvre and Block, 1992). When a reproductive management system that eliminates the need for estrous expression (Aréchiga et al., 1998; Britt and Gaska, 1998; Burke et al., 1996; De la Sota et al., 1998; Pursley et al., 1995; Pursley et al., 1997a, Pursley et al., 1997b; Schmitt et al., 1996; Vasconcelos et al., 1999) was used in

lactating cows, it was observed that administration of bST increased pregnancy rates to the synchronized service (Moreira et al., 2000d). This was a novel observation that needed to be replicated in further experiments. Moreover, it was also important to identify physiological windows influenced by bST to increase pregnancy rates.

The timed artificial insemination protocol (Ovsynch/TAI) is initiated with an injection of GnRH given at random stages of the estrous cycle followed 7 d later by an injection of $PGF_{2\alpha}$. At 48 h after injection of $PGF_{2\alpha}$, a second injection of GnRH is administered and cows are inseminated at approximately 16 h after GnRH without the need for estrous detection. Recent data indicated that stage of the estrous cycle at which the Ovsynch/TAI protocol is initiated affects subsequent pregnancy rates (Moreira et al., 2000b; Vasconcelos et al., 1999). Initiation of the Ovsynch/TAI protocol at the late luteal phase (i.e., day 13 to 17 of the estrous cycle) may lead to premature regression of the corpus luteum (CL), and cows are observed in estrus before the second injection of GnRH of the Ovsynch/TAI program (Moreira et al., 2000b). Premature CL regression may cause cows to ovulate before the time of artificial insemination, which may reduce chances for conception to the synchronized service (Moreira

et al., 2000b). Initiation of the Ovsynch/TAI protocol during the metestrus phase (i.e., day 1 to 4 of the estrous cycle) may lead to failure in the synchronization of a new follicular wave by the first GnRH injection. Such a failure may affect the ovulatory follicle and result in a CL that produces less progesterone (P_4) after ovulation induced by the second GnRH injection of the Ovsynch/TAI protocol (Moreira et al., 2000b), and compromise subsequent pregnancy rates (Moreira et al., 2000d). Furthermore, initiation of the Ovsynch/TAI protocol during the proestrous phase (i.e., days 18 to 21 of the cycle) may result in a failure of the injection of $PGF_{2\alpha}$ to completely regress the CL (Moreira et al., 2000b) and lower pregnancy rates after timed insemination (Moreira et al., 2000d). Collectively, these observations suggest that the optimal stage of the estrous cycle at which the Ovsynch/TAI protocol should be initiated corresponds to the early luteal phase (i.e., between day 5 and day 12 of the estrous cycle).

In order to target initiation of the Ovsynch/TAI protocol during the early luteal phase, it is necessary to pre-synchronize cows before administration of the first injection of GnRH. Use of two $PGF_{2\alpha}$ injections given 14 d apart constitutes a relative inexpensive and practical

system for estrous synchronization and has been used extensively in lactating dairy cows (Fergunson and Galligan, 1993). Such a program is expected to synchronize 90 to 95% of all cyclic cows to express estrus within a 7 d period. Thus, use of two $PGF_{2\alpha}$ injections to pre-synchronize cows to initiate the Ovsynch/TAI protocol at the targeted early luteal phase should increase subsequent pregnancy rates.

Objectives of the present experiment were to replicate the previous observation that bST increases pregnancy rates to the Ovsynch/TAI protocol; to observe whether bST increases pregnancy rates through its effects before or after timed insemination; and to compare pregnancy rates of cows that initiate the Ovsynch/TAI protocol at random stages of the cycle to cows that initiate the Ovsynch/TAI protocol at a targeted stage of the estrous cycle.

MATERIALS AND METHODS

The experiment was conducted on a commercial dairy located in northern Florida (Trenton, FL) during the months of February through June 1998. Lactating dairy cows were kept in free stall facilities and milked three times daily at approximately 8 h intervals with the first milking being initiated between 0800 and 1200. A total mixed ration

consisting of 33% forage (corn silage, alfalfa hay, and bermuda grass silage) and 67% concentrate (percentage of DM; ground corn, hominy, whole cottonseed, soybean meal, citrus pulp) containing 1.72 Mcal of NE_l/kg and 16.7% CP (percentage of DM) was fed throughout the experimental period. Cows were assigned randomly to initiate the experiment weekly based on day postpartum (PP), and all injections were kept at fixed days of the week. Before assignment, cows were blocked by month of synchronization (March and April for the first service; April/May and May/June for the second service) and by parity (primiparous versus multiparous). Cows diagnosed with any evident health problems such as toxic metritis, displaced abomasum, or acute mastitis immediately before or during the experiment were not included in the analysis. The experiment was designed as a 2 x 3 factorial experiment with two main factors: presence or absence of pre-synchronization and day of initiation of bST treatment.

At 37 ± 3 d PP, cows were assigned randomly to pre-synchronized or control groups. Cows receiving the pre-synchronization treatment (presynch) were injected with $PGF_{2\alpha}$ (Lutalyse, Pharmacia, Kalamazoo, MI; 25 mg, i.m.) and received a second injection of $PGF_{2\alpha}$ (25 mg, i.m.) 14 d later, at 51 ± 3 d PP. Cows in the control group received

no pre-synchronization treatment. All experimental cows initiated the Ovsynch/TAI protocol with an injection of GnRH (Cystorelin, Merial Co., Athens, GA; 100 μg, i.m.) at 12 d after the second injection of $PGF_{2\alpha}$ within the pre-synchronization treatment (63 ± 3 d PP). Cows were injected 7 d later with $PGF_{2\alpha}$ (40 mg, i.m.), and injected with a second injection of GnRH (100 μg, i.m.) at 48 h after injection of $PGF_{2\alpha}$. All experimental cows received their first service by timed insemination 16 to 20 h after the second GnRH injection, at 73 ± 3 d PP. Two sires of proven fertility were used randomly to inseminate experimental cows. Because approximately 90 to 95% of cyclic cows receiving the presynch treatment would be in estrus within 7 days of the second injection of $PGF_{2\alpha}$, the first injection of GnRH of the Ovsynch/TAI protocol was administered 12 d after the second injection of $PGF_{2\alpha}$ to target cows to initiate the protocol between days 5 to 11 of the estrous cycle. During the Ovsynch/TAI protocol, the dose of $PGF_{2\alpha}$ was increased from 25 to 40 mg in an attempt to decrease the incidence of cows having incomplete CL regression after injection of $PGF_{2\alpha}$.

Pregnancy was diagnosed initially at 32 d after timed insemination with an Aloka 500V ultrasound device (Aloka Co. Ltd., Tokyo, Japan) equipped with a 7.5 MHz linear-

array transrectal transducer to identify fetal fluid, appearance of fetus, and fetal heart beat. Cows diagnosed pregnant at ultrasonography were re-examined for pregnancy by rectal palpation at 74 d after timed insemination to determine embryonic losses. Cows diagnosed non-pregnant at 32 d after first-service timed insemination by ultrasonography were scheduled to re-initiate the Ovsynch/TAI protocol. Non-pregnant cows received an injection of GnRH (100 μg, i.m.), an injection of $PGF_{2\alpha}$ (40 mg, i.m.) 7 d later, a second injection of GnRH (100 μg, i.m.) 48 h after $PGF_{2\alpha}$, and were timed inseminated for their second service 16 to 20 h later (at 115 ± 3 d PP). At 32 d after the second timed insemination, cows were examined for pregnancy by ultrasound. Cows diagnosed pregnant at ultrasonography were re-examined by rectal palpation at 74 d after timed insemination. After the second service, non-pregnant cows returned to the dairy's traditional reproductive management system, which relied upon estrous detection for insemination. Cows diagnosed pregnant at 32 d after timed insemination that were later diagnosed non-pregnant at 74 d after timed insemination were considered to have had embryonic loss between 32 and 74 d of pregnancy.

The second main effect was initiation of bST treatment at 63 ± 3, 73 ± 3, or 147 ± 3 d PP. Once bST treatment (Posilac, Monsanto, St. Louis, MO; 500 mg, s.c.) was initiated, cows were re-injected with bST every 14 d thereafter until 30 d before the beginning of the dry period. A group of cows (bST-63) received their first injection of bST at 63 ± 3 d PP when the first GnRH dose was administered. A second group (bST-73) initiated bST treatment at 73 ± 3 d PP when cows received their first service as a timed insemination. A control group of cows received their first injection of bST only at 147 ± 3 d PP. Thus, cows in the control group were not affected by bST until after pregnancy rates for the second-service timed insemination were determined. Therefore, under the proposed factorial design, six experimental groups were created: control/bST-63 (n = 89), control/bST-73 (n = 88), control/control (n = 97), presynch/bST-63 (n = 90), presynch/bST-73 (n = 91), and presynch/control (n = 88).

At 63 ± 3 d PP (first injection of GnRH of the first-service timed insemination), body condition score (BCS) of cows were recorded. Body condition scores were given based on a 1 (thin) to 5 (obese) scale using a quarter point system (Edmonson et al., 1989) by two veterinarians who agreed upon a score.

Classification According to Plasma P_4 Concentrations

Blood samples were collected from all experimental cows at 51 ± 3 d PP (second $PGF_{2\alpha}$ injection of the presynch treatment), 63 ± 3 d PP (first GnRH injection of the first-service Ovsynch/TAI protocol), 70 ± 3 d PP (injection of $PGF_{2\alpha}$ of the Ovsynch/TAI protocol), 73 ± 3 d PP (second injection of the Ovsynch/TAI protocol), and at 80 ± 3 d PP (6 d after first service timed insemination). Blood samples were collected from the coccygeal vessel by venipuncture into heparinized tubes and kept on ice until centrifugation (2619 x *g* for 30 min). Plasma was separated and stored at - 20 °C until assayed for P_4 by single-antibody RIA (Knickerbocker et al., 1986). Sensitivity of the P_4 assay was 0.3 ng/mL, and the intrassay and interassay coefficients of variation were 13.8% and 5.9%, respectively.

Due to missing blood samples, not all experimental cows were included in the following classifications. Cows (n = 499; approximately 92% of all experimental cows) were classified as cyclic or anestrus at the initiation of the first-service Ovsynch/TAI protocol based on plasma P_4 concentrations of samples collected at 51 ± 3 d PP and at 63 ± 3 d PP. Cows with both plasma P_4 concentrations equal

or less than 1.0 ng/mL were classified as anestrus, whereas cows which had at least one plasma P_4 concentration greater than 1.0 ng/mL were considered to be cyclic at initiation of the first service Ovsynch/TAI protocol.

P_4-classes

Cows also were grouped into P_4-classes according to plasma P_4 concentrations of the two initial blood samples collected at the first GnRH injection at 63 ± 3 d PP and at the injection of $PGF_{2\alpha}$ administered 7 d later (Moreira et al., 2000d). At the first GnRH injection, cows were considered to have low plasma P_4 if concentrations were ≤ 1.0 ng/mL. Cows with plasma P_4 concentrations > 1.0 ng/mL at the first GnRH injection were considered to have a functional CL. However, cows in anestrus may respond to an injection of GnRH and luteinize follicles to produce plasma P_4 concentrations that will not exceed 2.5 ng/mL 7 d later (T. Kassa and W. W. Thatcher, 1998, unpublished observations). Therefore, anestrous cows can have plasma P_4 concentrations between 1.0 and 2.5 ng/mL 7 d after an injection of GnRH, even if an ovulation was not induced. Also, it is possible that cows initiating the Ovsynch/TAI protocol at proestrus may fail to ovulate to a GnRH injection and develop a luteinized follicle that produces plasma P_4 concentrations up to 2.5 ng/mL 7 d later. In this

manner, cows classified as cyclic based on high P_4 concentrations at 51 ± 3 d PP (> 1.0 ng/mL) but with low P_4 concentrations at 63 ± 3 d PP (≤ 1.0 ng/mL) may not ovulate a follicle in response to GnRH but, instead, develop a luteinized follicle that can produce plasma P_4 concentrations between 1.0 and 2.5 ng/mL 7 d later. Hence, cows were divided into four groups according to their plasma P_4 concentrations, as depicted in Table 3.1. Cows with high plasma P_4 concentrations (> 1.0 ng/mL) at the first injection of GnRH of the Ovsynch/TAI protocol had an active CL and, therefore, cut-off concentrations for plasma P_4 7 d later were maintained at 1.0 ng/mL. In contrast, cows with low plasma P_4 concentrations (≤ 1.0 ng/mL) at the first GnRH injection of the Ovsynch/TAI protocol were either anestrous or in the proestrous stage of the estrous cycle and, therefore, the cut-off concentrations for plasma P_4 7 d later were raised to 2.5 ng/mL.

Table 3.1. Classification of experimental cows based on plasma P_4 concentrations at the first injection of GnRH (d 0) and at the injection of $PGF_{2\alpha}$ (d 7) of the Ovsynch/TAI protocol.

P_4-classes	Plasma P_4 at GnRH (d 0)	Plasma P_4 at $PGF_{2\alpha}$ (d 7)
LOW-LOW	≤ 1.0 ng/mL	≤ 2.5 ng/mL
HIGH-LOW	> 1.0 ng/mL	≤ 1.0 ng/mL
LOW-HIGH	≤ 1.0 ng/mL	> 2.5 ng/mL
HIGH-HIGH	> 1.0 ng/mL	> 1.0 ng/mL

Cyclic cows classified as HIGH-HIGH were further subdivided into two categories. Plasma P_4 concentrations at the first GnRH between 1.0 and 2.5 ng/mL were considered to be medium concentrations, whereas plasma P_4 concentrations greater than 2.5 ng/mL were considered to be high concentrations. Our objective was to estimate the stage of the estrous cycle at initiation of the Ovsynch/TAI protocol. In this manner, cows that probably initiated the Ovsynch/TAI protocol at metestrus (plasma P_4 concentrations between 1.0 and 2.5 ng/mL at first GnRH) could be compared to cows that probably initiated the protocol during diestrus (plasma P_4 concentrations > 2.5 ng/mL at first GnRH). Accordingly, HIGH-HIGH cyclic cows were further classified as having medium or high plasma P_4 concentrations at the first GnRH injection of the Ovsynch/TAI protocol.

Similarly, cows classified as having HIGH plasma P_4 concentrations at the $PGF_{2\alpha}$ injection of the first-service Ovsynch/TAI protocol were classified according to their plasma P_4 concentrations of samples collected before the second injection of GnRH, at 48 h after injection of $PGF_{2\alpha}$. Cows classified as having LOW plasma P_4 concentrations at the injection of $PGF_{2\alpha}$ of the first-service Ovsynch/TAI protocol probably did not have a functionally active CL

that could respond to the luteolytic dose of $PGF_{2\alpha}$, and therefore, were not considered in this classification. Initially, cows were considered to have undergone complete CL regression if plasma P_4 concentrations were less than or equal to 1.0 ng/mL or to have incomplete CL regression if plasma P_4 concentrations were greater than 1.0 ng/mL 48 h after injection of $PGF_{2\alpha}$. After that, further analyses were conducted and cows were considered to have undergone complete CL regression if plasma P_4 concentrations were less than or equal to 2.0 ng/mL or to have incomplete CL regression if plasma P_4 concentrations were greater than 2.0 ng/mL 48 h after injection of $PGF_{2\alpha}$.

Furthermore, blood samples collected at 6 d after first-service timed insemination (80 ± 3 d PP) were used to classify cows as ovulating or not to the second GnRH injection of the first service Ovsynch/TAI. Only cows with completely regressed CL at 48 h after the injection of $PGF_{2\alpha}$ were used for this classification. Cows with plasma P_4 concentrations greater than 2.5 ng/mL were considered to have ovulated to the second injection of GnRH.

Alternative P_4-classes

Such a classification as described above has the disadvantage of being complex. Thus, an alternative

classification system was used (Table 3.2). Cut-off concentrations for plasma P_4 at $PGF_{2\alpha}$ (i.e., d 7) was maintained at 2.5 ng/mL regardless of whether cows had low or high plasma P_4 concentrations at the first GnRH injection of the Ovsynch/TAI protocol.

Table 3.2. Alternative classification of experimental cows based on plasma P_4 concentrations at the first injection of GnRH (d 0) and at the injection of $PGF_{2\alpha}$ (d 7) of the Ovsynch/TAI protocol.

P_4-classes	Plasma P_4 at GnRH (d 0)	Plasma P_4 at $PGF_{2\alpha}$ (d 7)
low-low	≤ 1.0 ng/mL	≤ 2.5 ng/mL
high-low	> 1.0 ng/mL	≤ 2.5 ng/mL
low-high	≤ 1.0 ng/mL	> 2.5 ng/mL
high-high	> 1.0 ng/mL	> 2.5 ng/mL

Cut-off concentrations for plasma P_4 utilized in further classifications such as whether cows were in the metestrus or diestrus at the first GnRH injection, whether cows regressed or not their CL completely after the injection of $PGF_{2\alpha}$, or whether cows ovulated or not after the second injection of GnRH of the Ovsynch/TAI protocol were not changed in subsequent analysis. Nonetheless, results do differ between the two analyses due to a different number of cows classified as LOW-HIGH and as HIGH-HIGH according to the original P_4-classes, and the number of cows classified as high-low and high-high according to the alternative P_4-classes.

Statistical Analysis

Data were analyzed using the method of least squares ANOVA using the General Linear Models procedure of the Statistical Analysis System software package (SAS, 1988). The mathematical model included the effects of treatment, month of synchronization, parity (i.e., primiparous or multiparous), technician performing the insemination, sire, barn, and all significant higher order interactions. BCS collected at 63 ± 3 d PP (first GnRH injection of the first-service Ovsynch/TAI protocol) was included as a continuous independent variable in the mathematical model.

Differences among experimental groups were determined by pre-established orthogonal contrasts. Contrasts examined the effects of pre-synchronization treatment (control/bST-63, control/bST-73, and control/control versus presynch/bST-63, presynch/bST73, and presynch/control), presence or absence of bST treatment (control/bST-63, control/bST-73, presynch/bST-63, and presynch/bST-73 versus control/control and presynch/control), and differences between groups receiving bST treatment at different times (control/bST-63 and presynch/bST-63 versus control/bST-73 and presynch/bST-73), as well as interactions between pre-synchronization and bST treatments.

Effects of cyclicity status (i.e., cyclic versus anestrous cows) on pregnancy rates, as well as its interaction with treatment groups, were later added to the mathematical model described above. Further analyses were conducted including only cyclic or anestrous cows. Effects of treatments on pregnancy rates of anestrous cows were not analyzed due to the low number of anestrous cows within each treatment group (approximately twenty anestrous cows per group). The effects of original P_4-classes (i.e., LOW-LOW, HIGH-LOW, LOW-HIGH, and HIGH-HIGH), alternative P_4-classes (i.e., low-low, high-low, low-high, and high-high), CL regression status (complete versus incomplete CL regression), and ovulation to the second injection of GnRH at first-service Ovsynch/TAI (ovulated versus non-ovulated) on pregnancy rates of cyclic cows were added sequentially to the described models in the respective order described above. Due to the fact that few cows were classified as LOW-LOW (or as low-low; n = 7), this category was excluded and orthogonal contrasts compared results from HIGH-LOW versus LOW-HIGH and HIGH-HIGH cows and LOW-HIGH versus HIGH-HIGH cows. Similar contrasts analyzed differences between high-low versus low-high and high-high cows and low-high versus high-high cows, according to the

alternative P_4-classes classification. Moreover, the frequency distribution of cows classified according to cyclicity status, P_4-classes, CL regression status, and ovulation to the second GnRH injection were analyzed among treatment groups. Differences were considered significant at a probability value of 0.05 or less.

RESULTS

First Service Reproductive Responses for all Experimental Cows

Pregnancy rates

Ten cows (seven at first service, three at second service) were diagnosed pregnant at ultrasonography but were later culled from the herd before pregnancy re-confirmation via rectal palpation. These cows were considered in the pregnancy rates analysis at 32 d after timed insemination, but were not included in the analyses for pregnancy losses and pregnancy rates at 74 d after timed insemination.

Analysis of pregnancy rates to the first synchronized service as diagnosed by ultrasonography at 32 and 74 d after insemination detected an interaction between bST treatment and pre-synchronization ($P < 0.01$; Table 3.3). This interaction was interpreted as a result of

increased pregnancy rates in pre-synchronized cows receiving bST compared to cows not treated with bST. However, no effect of bST treatment was detected when cows were not pre-synchronized.

Table 3.3. Pregnancy rates (PR) at 32 and at 74 d after first-service timed insemination for all experimental cows according to treatment groups (LSM ± SE).

Treatment groups	n [1]	PR at 32 d (%)	n [1]	PR at 74 d (%)
Control/bST-63	89	32.5 ± 6.1 [a]	89	29.4 ± 5.9 [a]
Control/bST-73	88	41.5 ± 5.9 [a]	87	28.1 ± 5.7 [a]
Control/control	97	36.0 ± 5.7 [a]	96	30.1 ± 5.5 [a]
Presynch/bST-63	90	52.8 ± 6.1 [b]	89	45.4 ± 5.8 [b]
Presynch/bST-73	91	55.5 ± 6.2 [b]	91	48.6 ± 6.0 [b]
Presynch/control	88	36.9 ± 6.0 [a]	84	33.6 ± 5.8 [a]

[1] Differences are due to 7 cows culled between 32 and 74 d after insemination.
[a,b] Different superscripts within columns indicate presynch by bST interaction ($P < 0.01$).

No differences in pregnancy rates were detected between cows injected with bST at 63 ± 3 d PP (first GnRH injection) or at 73 ± 3 d PP (timed insemination). Pregnancy rates at 32 d after insemination were influenced by BCS recorded at ultrasonography ($y = 4.19 + 16.2x$, where y = pregnancy rate and x = BCS; $P < 0.01$; $R^2_{model} = 0.074$ and $R^2_{reg} = 0.018$). Similarly, BCS recorded at ultrasonography was positively correlated with pregnancy rates at 74 d

after TAI (y = -21.0 + 18.0x, where y = pregnancy rate and x = BCS; $P < 0.01$; $R^2_{model} = 0.083$ and $R^2_{reg} = 0.024$).

Pregnancy losses

No differences were observed in pregnancy losses due to treatment effects: control/bST-63 = 9.5 ± 6.8%, control/bST-73 = 26.6 ± 6.2%, control/control = 16.2 ± 6.4%, presynch/bST-63 = 16.1 ± 5.6%, presynch/bST-73 = 11.0 ± 5.7%, and presynch/control = 6.9 ± 6.5%. Moreover, pregnancy losses were not affected by parity (i.e., primiparous versus multiparous), month of synchronization, sire, barn or BCS. Overall, pregnancy losses between 32 and 74 d after insemination were 13.6% (29/213).

Cyclic versus anestrus

A subset of the initial 543 experimental cows (n = 499) was classified as anestrus or cyclic according to plasma P_4 concentrations collected at 51 ± 3 and at 63 ± 3 d PP. Among these cows, 77% (382/499) were classified as cyclic and 23% (117/499) were classified as anestrus at initiation of the first service Ovsynch/TAI protocol. No differences in the frequency of cows classified as anestrus were detected among treatment groups. Incidence of anestrus was greater ($P < 0.01$) in primiparous than in multiparous cows (37.3 ± 3.1% > 15.7 ± 2.2%, respectively). Furthermore, a quadratic effect of body condition score at

63 ± 3 d PP (range = 2.0 to 4.5) on the frequency of cows classified as anestrus indicated that incidence of anestrus decreased as body condition scores increased (Figure 3.1).

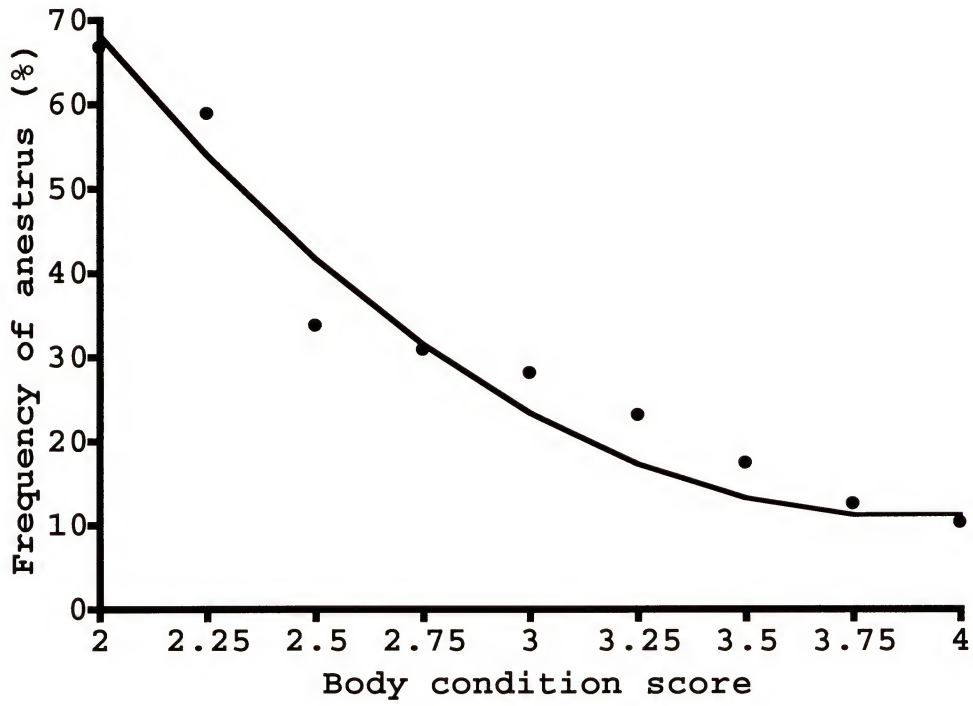

Figure 3.1. Percentage of cows classified as anestrus according to body condition scores at first injection of GnRH of the first-service Ovsynch/TAI. Quadratic effect of body condition scores on the incidence of anestrus was significant ($P < 0.01$). $R^2_{model} = 0.12$ and $R^2_{reg} = 0.077$. Black circles represent actual means.

When the effect of cyclic status was added to the mathematical models, it was observed that pregnancy rates, as diagnosed at 32 and at 74 d after insemination, were decreased ($P < 0.01$) in cows which initiated the Ovsynch/TAI protocol in anestrus (24.6 ± 4.9% and 22.4 ± 4.8%, respectively) compared to cows classified as cyclic

(50.1 ± 3.4% and 41.7 ± 3.3%, respectively). Moreover, an interaction between treatment and cyclicity status was observed ($P < 0.01$). Therefore, cows were subsequently divided into either anestrous or cyclic cows and first service pregnancy rates were evaluated.

First Service Reproductive Responses for Cyclic Cows

Pregnancy rates

When pregnancy rates at 32 d after insemination were analyzed to include only cows classified as cyclic, results indicated both an effect of bST ($P < 0.01$) and pre-synchronization ($P < 0.01$) treatments (Table 3.4). Similar results were obtained when first service pregnancy rates at 74 d after insemination for cyclic cows were examined. Both bST treatments ($P < 0.04$) and pre-synchronization ($P < 0.01$) increased first-service pregnancy rates Table 3.4).

No differences were detected in pregnancy rates between cows treated with bST at 63 ± 3 or at 73 ± 3 d PP. Results indicate that bST treatment, regardless of when it was initiated, increased first-service pregnancy rates when cyclic cows were not pre-synchronized and also when cows were pre-synchronized. Results also indicated that pre-synchronized cows had increased first-service pregnancy rates compared to cows that initiated the Ovsynch/TAI

protocol at random stages of the estrous cycle. A trend (P = 0.07) for an interaction between bST treatments and pre-synchronization was observed for pregnancy rates at 32 and 74 d after insemination and may indicate that the increase in pregnancy rates due to use of bST was of a greater magnitude when cows were pre-synchronized. No effects of BCS were observed at 32 d pregnancy rates of cyclic cows, but BCS was positively associated with pregnancy rates at 74 d after insemination for cyclic cows ($y = -1.11 + 14.75x$, where y = pregnancy rate and x = BCS; $P < 0.02$; $R^2_{model} = 0.11$ and $R^2_{reg} = 0.015$).

Table 3.4. Pregnancy rates (PR) at 32 and at 74 d after first-service timed insemination for cyclic cows according to treatment groups (LSM ± SE).

Treatment groups	n [1]	PR at 32 d (%)	n [1]	PR at 74 d (%)
Control/bST-63	63	39.1 ± 7.4 [ax]	63	34.2 ± 7.2 [aw]
Control/bST-73	67	50.6 ± 7.2 [ax]	66	33.7 ± 7.0 [aw]
Control/control	67	34.4 ± 7.0 [ay]	66	25.3 ± 6.9 [az]
Cresynch/bST-63	59	67.8 ± 7.3 [bx]	58	58.2 ± 7.1 [bw]
Cresynch/bST-73	60	63.5 ± 7.3 [bx]	60	56.1 ± 7.2 [bw]
Cresynch/control	66	46.9 ± 6.9 [by]	62	42.6 ± 6.8 [bz]

[1] Differences are due to 7 cows culled between 32 and 74 d after insemination.
[a,b] Different superscripts within columns indicate presynch effect ($P < 0.01$).
[x,y] Different superscripts within columns indicate bST effect ($P < 0.01$).
[w,z] Different superscripts within columns indicate bST effect ($P < 0.04$).

Pregnancy losses

Overall, pregnancy losses between 32 and 74 d after insemination were 14.6% (26/178). Pregnancy losses among cyclic cows were not affected by treatment groups: control/bST-63 = 10.5 ± 7.7%, control/bST-73 = 26.2 ± 6.9%, control/control = 24.1 ± 8.2%, presynch/bST-63 = 16.1 ± 6.3%, presynch/bST-73 = 10.3 ± 6.6%, and presynch/control = 7.8 ± 7.1%. However, a trend ($P < 0.10$) may indicate that cows pre-synchronized had less pregnancy losses than cows not pre-synchronized (11.6 ± 3.8% < 21.2 ± 4.3%). Pregnancy losses were not affected by parity (i.e., primiparous versus multiparous), month of synchronization, sire, barn, or BCS.

Results Based on Original P_4-Classes

Original P_4-classes

Among cyclic cows (n = 382), 366 cows were categorized according to P_4-classes. Frequency distribution and pregnancy rates for P_4-classes are depicted in Table 3.5. Because of the low number of LOW-LOW cows (n = 7), that category was not included in further analyses. Frequency distribution of cyclic cows among the remaining P_4-classes was affected by pre-synchronization (Table 3.6).

Table 3.5. Frequency distribution (Freq) and pregnancy rates (PR) at 32 and at 74 d after first-service insemination (LSM ± SE) for cyclic cows classified according to P_4-classes.

P_4-classes	n	Freq	PR at 32 d (%)	PR at 74 d (%)
LOW-LOW [1]	7	1.9%	28.6	14.3
HIGH-LOW	36	9.8%	22.8 ± 8.8 [a]	19.4 ± 8.8 [a]
LOW-HIGH	22	6.0%	67.7 ± 10.9 [b]	55.1 ± 10.7 [b]
HIGH-HIGH	301	82.2%	51.3 ± 4.1 [b]	43.7 ± 4.0 [b]

[1] Due to small numbers, LOW-LOW cows were not included in the analyses.
[a,b] Different superscripts within columns indicate significant contrasts ($P < 0.01$).

Table 3.6. Frequency of cyclic cows classified as HIGH-LOW, LOW-HIGH, and HIGH-HIGH among treatment groups (LSM ± SE).

Treatment groups	HIGH-LOW (%)	LOW-HIGH (%)	HIGH-HIGH (%)
Control/bST-63	12.0 ± 3.7 [a]	7.5 ± 2.9 [a]	80.1 ± 4.6 [a]
Control/bST-73	12.3 ± 3.7 [a]	9.6 ± 3.0 [a]	74.3 ± 4.6 [a]
Control/control	22.2 ± 3.7 [a]	13.1 ± 2.9 [a]	61.1 ± 4.5 [a]
Presynch/bST-63	5.0 ± 4.0 [b]	1.5 ± 3.2 [b]	92.8 ± 5.0 [b]
Presynch/bST-73	4.8 ± 4.0 [b]	4.8 ± 3.2 [b]	88.0 ± 5.0 [b]
Presynch/control	7.7 ± 3.7 [b]	6.4 ± 3.0 [b]	82.1 ± 4.7 [b]

[a,b] Different superscripts within columns indicate presynch effect ($P < 0.01$).

Treatment effects on pregnancy rates in HIGH-LOW and LOW-HIGH P_4-classes were not examined due to small numbers of cows within these categories. However, effects of

treatments on pregnancy rates for HIGH-HIGH cyclic cows were examined and results are depicted in Table 3.7.

Table 3.7. Pregnancy rates (PR) at 32 and at 74 d after first-service insemination for cyclic cows classified as HIGH-HIGH among treatment groups (LSM ± SE).

Treatment groups	n [1]	PR at 32 d (%)	n [1]	PR at 74 d (%)
Control/bST-63	52	44.9 ± 8.5 [ay]	52	39.9 ± 8.3 [a]
Control/bST-73	52	38.1 ± 9.8 [ay]	51	34.8 ± 9.7 [a]
Control/control	42	41.5 ± 9.1 [ay]	41	32.1 ± 9.1 [a]
Presynch/bST-63	52	69.2 ± 8.0 [bx]	51	62.6 ± 8.0 [b]
Presynch/bST-73	48	64.8 ± 8.5 [bx]	48	57.3 ± 8.4 [b]
Presynch/control	55	50.7 ± 7.7 [by]	52	47.7 ± 7.7 [b]

[1] Differences are due to 6 cows culled between 32 and 74 d after insemination.
[a,b] Different superscripts within columns indicate presynch effect ($P < 0.01$).
[x,y] Different superscripts within columns indicate presynch by bST interaction ($P < 0.03$).

Because an effect of pre-synchronization was observed among cyclic cows classified as HIGH-HIGH, plasma P_4 concentrations at the first GnRH injection and at the injection of $PGF_{2\alpha}$ 7 d later were analyzed. At the first GnRH injection, no differences in plasma P_4 concentrations were observed among treatment groups, but plasma P_4 concentrations at the injection of $PGF_{2\alpha}$ were greater ($P < 0.01$) for pre-synchronized cows (Figure 3.2).

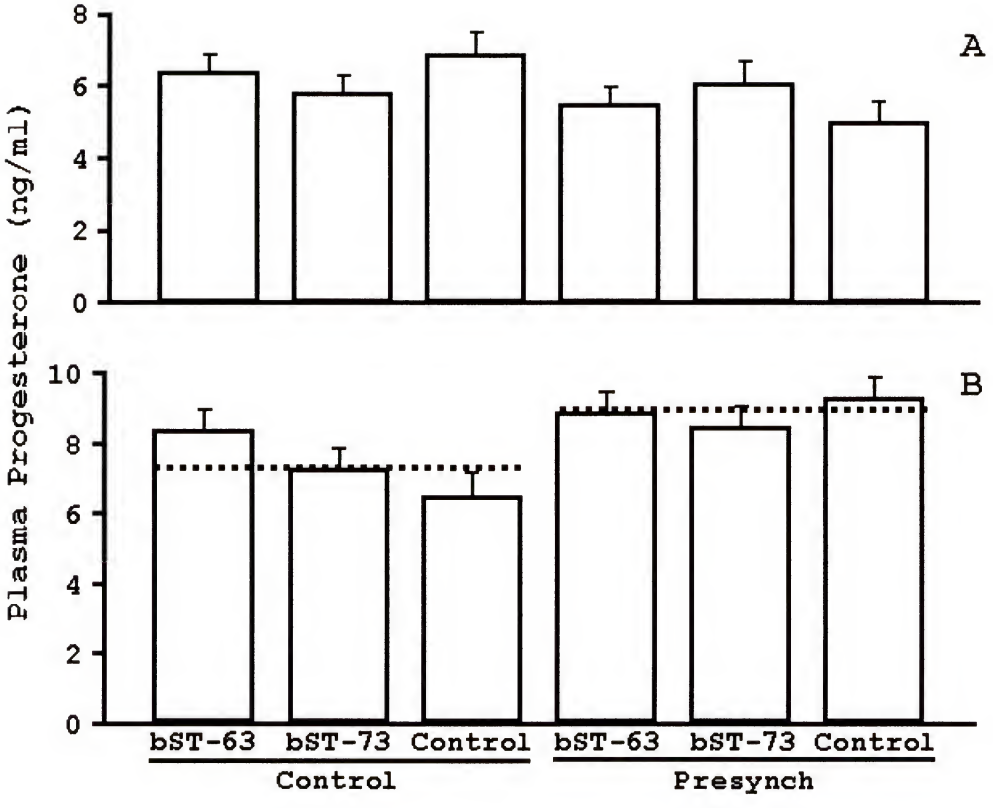

Figure 3.2. Plasma progesterone concentrations at first injection of GnRH (Panel A) and at injection of $PGF_{2\alpha}$ (Panel B) for cyclic cows classified as HIGH-HIGH according to treatment groups. Orthogonal contrasts indicated that plasma P_4 concentrations at $PGF_{2\alpha}$ injection were increased for pre-synchronized cows compared to cows not pre-synchronized (8.9 ± 0.4 ng/mL > 7.5 ± 0.4 ng/mL; $P < 0.01$) as represented by doted lines.

Plasma concentrations of P_4 at $PGF_{2\alpha}$ were added to the mathematical models for pregnancy rates of HIGH-HIGH cows. A trend ($P < 0.08$) for a positive association between plasma P_4 concentrations at $PGF_{2\alpha}$ and pregnancy rates was detected at 32 d after first-service timed insemination. At 74 d after insemination, plasma P_4 concentrations were

positively associated with increased pregnancy rates (y = 1.02 + 1.83x, where y = pregnancy rate and x = plasma P_4 concentration, ng/mL; $P < 0.01$; $R^2_{model} = 0.13$ and $R^2_{reg} = 0.026$).

In addition, HIGH-HIGH cyclic cows were further subdivided into two groups based on plasma P_4 at first GnRH injection of the Ovsynch/TAI protocol: cows estimated to have initiated the protocol at metestrus (> 1.0 and ≤ 2.5 ng/mL at GnRH; n = 74) and cows estimated to have initiated the protocol at diestrus (> 2.5 ng/mL at GnRH; n = 227). Pregnancy rates at 32 d after insemination were increased ($P < 0.01$) for cows estimated to have initiated the Ovsynch/TAI protocol during diestrus (53.7 ± 4.1%) compared with cows estimated to have initiated the protocol during metestrus (34.5 ± 6.1%). Similarly, at 74 d after Ovsynch/TAI, pregnancy rates were greater ($P < 0.01$) for cows in the diestrus than for cows in the metestrus (50.0 ± 4.1% > 26.5 ± 6.2%, respectively).

CL regression status

Among cyclic cows classified as LOW-HIGH and HIGH-HIGH (n = 323), who potentially could respond to an injection of $PGF_{2\alpha}$, cows (n = 322) were classified as having complete or incomplete CL regression. When cows were considered to

have incomplete CL regression if plasma P_4 was greater than 1.0 ng/mL at 48 h after the injection of $PGF_{2\alpha}$, results indicated that 38.5% of the cows (124/322) had not completely regressed their CL. No differences were observed in pregnancy rates of cows that failed to completely regress their CL compared to cows with complete CL regression at 32 d (52.1 ± 5.7% and 54.8 ± 4.6%, respectively) and at 74 d after insemination (48.0 ± 5.6% and 44.5 ± 4.6%, respectively).

Results were re-analyzed with cows being considered to have had incomplete CL regression if plasma P_4 concentrations at 48 h after $PGF_{2\alpha}$ were greater than 2.0 ng/mL. Only 6.2% of cows (20/322) had plasma P_4 concentrations greater than 2.0 ng/mL and were classified as having incomplete CL regression. Incidence of cows having incomplete CL regression was not affected by treatment, parity, month of synchronization, or P_4-classes. Pregnancy rates at 32 and at 74 d after insemination for cows having incomplete CL regression were 20.0% (4/20), which were lower than pregnancy rates of cows having complete CL regression at 32 d after TAI (55.0 ± 4.1%; $P < 0.01$) and also at 74 d after TAI (46.7 ± 4.1%; $P < 0.04$).

Ovulation to second GnRH injection

Cyclic cows with high P_4 concentrations at $PGF_{2\alpha}$ injection (i.e., LOW-HIGH and HIGH-HIGH) and that experienced complete CL regression CL 48 h later (plasma P_4 ≤ 2.0 ng/mL) were further classified as ovulating or not to the second GnRH injection of the Ovsynch/TAI protocol (n = 295). Approximately 8.8% (26/295) of the cows were classified as failing to ovulate to the second GnRH injection of the Ovsynch/TAI protocol. Treatment, parity, month of synchronization and P_4-Classes (i.e., LOW-HIGH and HIGH-HIGH) did not influence the percentage of cows failing to ovulate to the second GnRH injection of the Ovsynch/TAI protocol. Among the latter group, 11.5% (3/26) were diagnosed pregnant at 32 d after TAI, but only one cow (3.8%, 1/26) was re-confirmed pregnant at 74 d after insemination. Plasma P_4 concentration at 6 d after insemination for that particular cow was 2.36 ng/mL. Therefore, pregnancy rates were reduced for cows classified as failing to ovulate to the second GnRH injection of the Ovsynch/TAI protocol ($P < 0.01$) compared with pregnancy rates of cows classified as ovulating to the second GnRH injection as diagnosed at 32 and 74 d after insemination (60.2 ± 4.2% and 51.9 ± 4.2%, respectively).

Plasma P_4 concentrations at 6 d after insemination were analyzed for cyclic cows classified as LOW-HIGH or as HIGH-HIGH which completely regressed their CL (based on a 2.0 ng/mL cut-off) and were considered to have ovulated after the second GnRH injection of the Ovsynch/TAI protocol (n = 269). No differences among treatments were observed (overall mean = 5.4 ng/mL). Plasma P_4 concentrations at 6 d after insemination also did not differ between cows classified as LOW-HIGH (5.5 ± 0.5 ng/mL, n = 17) or as HIGH-HIGH (5.4 ± 0.1 ng/mL, n = 252). Plasma P_4 concentrations at 6 d after insemination were similar between cows later diagnosed pregnant (5.4 ± 0.3 ng/mL, n = 156) or nonpregnant (5.5 ± 0.3 ng/mL, n = 113) at ultrasonography.

Results Based on Alternative P_4-Classes

Alternative P_4-classes

Among cyclic cows (n = 382), 366 cows were categorized according to P_4-classes. Frequency distribution and pregnancy rates for P_4-classes are depicted in Table 3.8. Analyses did not include low-low cows (n = 7). Frequency distribution of cyclic cows among the remaining P_4-classes was affected by pre-synchronization (Table 3.9).

Table 3.8. Frequency distribution (Freq) and pregnancy rates (PR) at 32 and at 74 d after first-service insemination (LSM ± SE) for cyclic cows classified according to P_4-classes.

P_4-classes	n	Freq	PR at 32 d (%)	PR at 74 d (%)
low-low [1]	7	1.9%	28.6	14.3
high-low	72	19.7%	34.8 ± 6.7 [a]	28.9 ± 6.6 [c]
low-high	22	6.0%	67.9 ± 11.0 [b]	55.2 ± 10.8 [d]
high-high	265	72.4%	51.8 ± 4.3 [b]	44.4 ± 4.2 [d]

[1] Due to small numbers, LOW-LOW cows were not included in the analyses.
[a,b] Different superscripts within columns indicate significant contrasts ($P < 0.01$).
[c,d] Different superscripts within columns indicate significant contrasts ($P < 0.03$).

Table 3.9. Frequency of cyclic cows classified as high-low, low-high, and high-high among treatment groups (LSM ± SE).

Treatment groups	high-low (%)	low-high (%)	high-high (%)
control/bST-63	7.4 ± 2.9 [a]	20.0 ± 4.9 [a]	72.1 ± 5.4 [a]
control/bST-73	9.6 ± 3.0 [a]	23.1 ± 4.9 [a]	63.5 ± 5.4 [a]
control/control	13.1 ± 2.9 [a]	39.2 ± 4.9 [a]	44.1 ± 5.3 [a]
presynch/bST-63	1.5 ± 3.2 [b]	10.8 ± 5.4 [b]	87.1 ± 5.8 [b]
presynch/bST-73	4.8 ± 3.2 [b]	16.4 ± 5.4 [b]	76.5 ± 5.9 [b]
presynch/control	6.4 ± 3.0 [b]	14.2 ± 5.0 [b]	75.6 ± 5.4 [b]

[a,b] Different superscripts within columns indicate presynch effect ($P < 0.01$).

Treatment effects pregnancy rates for HIGH-HIGH cyclic cows were examined (Table 3.10). Pregnancy rates were higher for cows pre-synchronized, and, among pre-

synchronized groups, bST treatment increased pregnancy rates at 32 d after timed insemination.

Table 3.10. Pregnancy rates (PR) at 32 and at 74 d after first-service insemination for cyclic cows classified as high-high among treatment groups (LSM ± SE).

Treatment groups	n [1]	PR at 32 d (%)	n [1]	PR at 74 d (%)
control/bST-63	47	45.2 ± 8.6 [ay]	47	39.9 ± 8.5 [a]
control/bST-73	45	43.8 ± 11.6 [ay]	44	38.8 ± 11.5 [a]
control/control	31	40.7 ± 10.0 [ay]	30	34.1 ± 10.1 [a]
presynch/bST-63	49	69.6 ± 8.5 [bx]	48	62.0 ± 8.4 [b]
presynch/bST-73	42	65.7 ± 9.0 [bx]	42	58.7 ± 8.9 [b]
presynch/control	51	46.9 ± 8.1 [by]	48	43.0 ± 8.1 [b]

[1] Differences are due to 6 cows culled between 32 and 74 d after insemination.
[a,b] Different superscripts within columns indicate presynch effect ($P < 0.02$).
[x,y] Different superscripts within columns indicate presynch by bST interaction ($P < 0.06$).

Plasma P_4 concentrations at the first GnRH injection and at the injection of $PGF_{2\alpha}$ administered 7 d later were analyzed for high-high cows. At the first GnRH injection of the Ovsynch/TAI protocol, no differences in plasma P_4 concentrations were observed among treatment groups (Figure 3.3, Panel A). However, plasma P_4 concentrations at the injection of $PGF_{2\alpha}$ were greater ($P < 0.01$) for pre-synchronized cows compared with cows not pre-synchronized (Figure 3.3, Panel B).

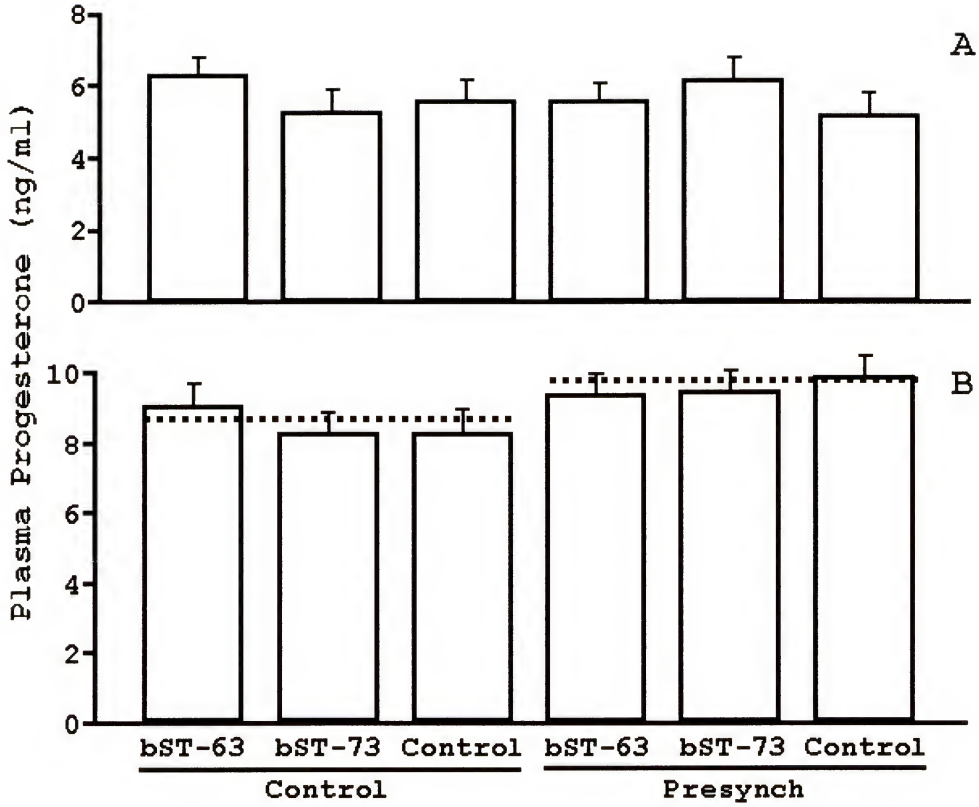

Figure 3.3. Plasma progesterone concentrations at first injection of GnRH (Panel A) and at injection of $PGF_{2\alpha}$ (Panel B) for cyclic cows classified as high-high according to treatment groups. Orthogonal contrasts indicated that plasma P_4 concentrations at $PGF_{2\alpha}$ injection were greater for pre-synchronized cows than for cows not pre-synchronized (9.6 ± 0.4 ng/mL > 8.7 ± 0.4 ng/mL; $P < 0.05$) as represented by doted lines.

Plasma concentrations of P_4 at $PGF_{2\alpha}$ were added to the mathematical models for pregnancy rates of high-high cows. A positive association ($P < 0.05$) between plasma P_4 concentrations at $PGF_{2\alpha}$ and pregnancy rates was detected at 32 d after insemination. At 74 d after insemination, plasma P_4 concentrations were positively associated with

increased pregnancy rates ($y = -0.19 + 2.36x$, where y = pregnancy rate and x = plasma P_4 concentration, ng/mL; $P < 0.01$; $R^2_{model} = 0.15$ and $R^2_{reg} = 0.037$).

Cyclic cows classified as high-high were further subdivided into two groups and classified as having medium (> 1.0 and ≤ 2.5 ng/mL; metestrus; n = 62) or high (> 2.5 ng/mL; diestrus; n = 203) plasma P_4 concentrations at first GnRH. Frequency distribution of cows among these categories was not influenced by treatment. Pregnancy rates at 32 d after insemination were increased ($P < 0.04$) for cows estimated to have initiated the Ovsynch/TAI protocol during diestrus (55.7 ± 5.1%) compared with cows estimated to have initiated the protocol during metestrus (40.1 ± 7.5%). At 74 d after insemination, greater pregnancy rates ($P < 0.01$) were observed for cows estimated to have initiated the Ovsynch/TAI protocol at diestrus as compared to cows estimated to have initiated the protocol at metestrus (50.5 ± 5.0% > 31.3 ± 7.5%).

CL regression status

Among cyclic cows classified as low-high and high-high that potentially could respond to an injection of $PGF_{2\alpha}$ (n = 287), cows were classified as having complete or incomplete CL regression (n = 286). When cows were considered to have incomplete CL regression due to plasma P_4 concentrations

being greater than 1.0 ng/mL at 48 h after the injection of $PGF_{2\alpha}$, 40.9% of the cows (117/286) had not completely regressed their CL. No differences were observed in pregnancy rates of cows that failed to undergo complete CL regression compared to cows with complete CL regression at 32 d (52.6 ± 5.8% and 55.6 ± 4.9%, respectively) and at 74 d after insemination (48.1 ± 5.8% and 44.7 ± 4.9%, respectively). Results were re-analyzed with cows being considered to have had incomplete CL regression if plasma P_4 concentrations at 48 h after $PGF_{2\alpha}$ were greater than 2.0 ng/mL. Only 7.0% of cows (20/286) had plasma P_4 concentrations greater than 2.0 ng/mL and were classified as having incomplete CL regression. Pregnancy rates at 32 and at 74 d after insemination for cows having incomplete CL regression were 20.0% (4/20), which were lower than pregnancy rates at 32 d after insemination (55.9 ± 4.3%; $P < 0.01$) and also at 74 d after insemination (47.2 ± 4.1%; $P < 0.03$) for cows having complete CL regression.

Ovulation to second GnRH injection

Cyclic cows with high P_4 concentrations at $PGF_{2\alpha}$ injection (i.e., low-high and high-high) and that completely regressed their CL 48 h later (plasma $P_4 \leq 2.0$ ng/mL) were further classified as ovulating or not to the

second GnRH injection of the Ovsynch/TAI protocol (n = 261). Approximately 9.2% (24/261) of the cows were classified as failing to ovulate to the second GnRH injection of the Ovsynch/TAI protocol. Treatment, parity, month of synchronization and P_4-Classes (i.e., low-high and high-high) did not influence the percentage of cows failing to ovulate to the second GnRH injection of the Ovsynch/TAI protocol. Among cows categorized as not ovulating, 12.5% (3/24) were diagnosed pregnant at 32 d after insemination, but only one cow (4.2%, 1/24) was re-confirmed pregnant at 74 d after insemination. Plasma P_4 concentration at 6 d after insemination for the latter cow was 2.36 ng/mL. Therefore, pregnancy rates were reduced for cows classified as failing to ovulate to the second GnRH injection of the Ovsynch/TAI protocol ($P < 0.01$) compared with pregnancy rates of cows classified as ovulating to the second GnRH injection at 32 and 74 d after insemination (61.1 ± 4.4% and 52.4 ± 4.4%, respectively).

Plasma P_4 concentrations at 6 d after insemination were analyzed for cyclic cows that ovulated after the second GnRH injection of the Ovsynch/TAI protocol (n = 231). These cows were classified as low-high or as high-high, had completely regressed their CL (based on a 2.0

ng/mL cut-off) and were considered to have ovulated to the second GnRH injection. No differences among treatments were observed for plasma P_4 concentrations (overall mean = 5.3 ng/mL). Plasma P_4 concentrations at 6 d after insemination also did not differ between cows classified as low-high (5.5 ± 0.4 ng/mL) or as high-high (5.1 ± 0.1 ng/mL). In addition, plasma P_4 concentrations at 6 d after insemination were similar between cows later diagnosed pregnant (5.3 ± 0.3 ng/mL) or nonpregnant (5.3 ± 0.3 ng/mL) at ultrasonography.

First Service Reproductive Responses for Anestrous Cows

Among cows classified as anestrus, mean pregnancy rates as diagnosed at 32 d after insemination were as follows: control/bST-63 = 15.8% (3/19), control/bST-73 = 12.5% (2/16), control/control = 39.1% (9/23), presynch/bST-63 = 26.1% (6/23), presynch/bST-73 = 15.0% (3/20), and presynch/control = 18.8% (3/16). When cows were re-examined by rectal palpation at 74 d after insemination, mean pregnancy rates were: control/bST-63 = 15.8% (3/19), control/bST-73 = 6.3% (1/16), control/control = 39.1% (9/23), presynch/bST-63 = 21.7% (5/23), presynch/bST-73 = 15.0% (3/20), and presynch/control = 18.8% (3/16). Overall pregnancy rates for anestrous cows at 32 d after

insemination was 22.2% (26/117) and 20.5% (24/117) at 74 d after insemination. Thus, pregnancy losses between 32 and 74 d after insemination were 7.7% (2/26) for anestrous cows.

Anestrous cows (n = 112) were further classified according to P_4-classes. Results indicated that 56 cows (50.0%) ovulated to the first injection of GnRH of the Ovsynch/TAI protocol, as indicated by P_4 concentrations > 2.5 ng/mL 7 d later (i.e., LOW-HIGH cows), whereas 56 cows (50.0%) failed to respond to the first GnRH injection and were classified as LOW-LOW.

When cows were classified as having either complete or incomplete CL regression based on a 2.0 ng/mL plasma P_4 concentration, only 3.6% (2/55) of LOW-HIGH cows had incomplete CL regression. Neither of the two cows classified as having incomplete CL regression were diagnosed pregnant at 32 and 74 d after insemination.

Among LOW-HIGH cows that completely regressed their CL upon injection of $PGF_{2\alpha}$ (n = 45), 90.5 ± 5.9% were classified as ovulating to the second injection of GnRH of the Ovsynch/TAI protocol, whereas ovulation rates for LOW-LOW cows (n = 54) were 49.1 ± 6.0% ($P < 0.01$). Pregnancy rates for cows ovulating to the second GnRH were higher (P

< 0.01) for LOW-HIGH cows than for LOW-LOW cows at 32 d (43.8 ± 6.5% > 12.0 ± 9.0%) and 74 d after TAI (39.6 ± 6.4% > 12.0 ± 8.9%). Only 8.9% of LOW-HIGH cows that had complete CL regression (4/45) failed to ovulate to the second injection of GnRH and none of those cows conceived to the timed insemination. Among LOW-LOW cows, 51.8% (28/54) were classified as having failed to respond to the second GnRH injection. Among the 28 cows not ovulating to the second GnRH injection of the Ovsynch/TAI protocol, only one cow (3.6%), which was on the borderline of being classified as ovulating to the second GnRH (plasma P_4 at 6 d after insemination was 2.4 ng/mL), conceived to the timed insemination. Overall, the Ovsynch/TAI protocol was able to induce cyclicity in 74.5% (82/110) of anestrous cows, based upon the number of anestrous cows which were classified as responding to either the first and/or second injection of GnRH.

Second Service Pregnancy Rates

Second service pregnancy rates were not affected by treatments as diagnosed at 32 and 74 d after insemination (Table 3.14). Cows that received their second service in May/June tended (P < 0.10) to have lower pregnancy rates at 32 d after insemination (19.9 ± 4.5%; n = 98) than cows

that received their second service in April/May (28.8 ± 3.1%; n = 184). Similarly, cows receiving their second service in May/June had lower (P < 0.06) pregnancy rates at 74 d after insemination (14.1 ± 4.1%; n = 97) than cows that received their second service in April/May (23.7 ± 2.9%; n = 182).

Table 3.14. Pregnancy rates (PR) at 32 and at 74 d after second-service insemination for all experimental cows among treatment groups (LSM ± SE).

Treatment groups	n [1]	PR at 32 d (%)	n [1]	PR at 74 d (%)
control/bST-63	57	28.4 ± 5.7	56	21.2 ± 5.4
control/bST-73	44	16.5 ± 6.4	44	14.1 ± 5.9
control/control	50	31.2 ± 6.1	50	24.5 ± 5.6
presynch/bST-63	42	22.9 ± 6.7	41	17.8 ± 6.2
presynch/bST-73	44	27.6 ± 6.4	43	19.0 ± 6.0
presynch/control	45	19.7 ± 6.5	45	16.9 ± 6.0

[1] Differences are due to 3 cows culled between 32 and 74 d after insemination.

When cyclicity status at initiation of the first-service Ovsynch/TAI was added to mathematical models, pregnancy rates to the second service diagnosed via ultrasonography at 32 d after insemination did not differ for cows classified as anestrus (23.1 ± 4.8%; n = 82) or cyclic (25.1 ± 3.5%; n = 174), and no interaction between cyclicity status and experimental treatments were observed

at this time. Similarly, no differences were observed in second service pregnancy rates at 74 d after insemination between cows classified as cyclic (20.1 ± 3.3%; n = 172) or anestrus (17.5 ± 4.5%; n = 81) at initiation of the first-service Ovsynch/TAI.

Pregnancy Rates at 115 d PP

Analyses of final pregnancy rates after the two synchronized services indicated an interaction ($P < 0.01$) between pre-synchronization and absence or presence of bST treatment. Among pre-synchronized cows, use of bST increased pregnancy rates at 115 d PP (presynch/bST-63 = 58.5 ± 6.4%, n = 82; presynch/bST-73 = 56.8 ± 6.5%, n = 87) compared with control (presynch/control = 43.4 ± 6.7%, n = 77). However, differences in pregnancy rates at 115 d PP were not observed when cows were not pre-synchronized (control/bST-63 = 42.7 ± 6.4%, n = 82; control/bST-73 = 36.4 ± 6.4%, n = 81; control/control = 48.9 ± 6.2%, n = 83).

After cyclicity status was added to the analysis, results indicated that cows classified as anestrus at initiation of the first Ovsynch/TAI protocol had lower ($P < 0.01$) pregnancy rates at 115 d PP (36.0 ± 5.4%, n = 107) than cows classified as cyclic (52.7 ± 3.6%, n = 350). No

significant interaction between treatments and cyclicity status was detected.

Late Pregnancy Loss and Calving Difficulty Scores

A total of 246 cows were diagnosed pregnant at 74 d after insemination after both first and second services. Approximately 14.2% of the former group (35/246) were culled from the herd before calving and, therefore, were excluded form the subsequent analyses.

The incidence of pregnancy losses after pregnancy diagnosis at 74 d after insemination was 8.0% (17/211). No effects of treatment groups on the incidence of late term pregnancy losses were detected: control/bST-63 = 9.1% (3/33), control/bST-73 = 11.1% (3/27), control/control = 8.1% (3/37), presynch/bST-63 = 5.1% (2/39), presynch/bST-73 = 10.0% (4/40), and presynch/control = 5.5% (2/35).

Only 4.6% of cows calving required assistance at parturition (9/194). No differences due to treatment were observed: control/bST-63 = 6.6% (2/30), control/bST-73 = 4.3% (1/23), control/control = 2.8% (1/35), presynch/bST-63 = 2.7% (1/37), presynch/bST-73 = 5.5% (2/36), and presynch/control = 6.0% (2/33).

DISCUSSION

Results indicated that both pre-synchronization and bST treatments, regardless of when it was initiated, increased pregnancy rates to the Ovsynch/TAI protocol. When all experimental cows were included in the analyses, first-service pregnancy rates of cows pre-synchronized and treated with bST were increased compared with control cows that were not under the effects of bST. In a previous study (Moreira et al., 2000d), it was hypothesized that bST could have increased pregnancy rates due to its effects before insemination on follicular and CL development that occurs during the Ovsynch/TAI protocol, and also due to its effects after insemination. If bST increased pregnancy rates due to its effects before insemination, it would be anticipated that pregnancy rates would be greater in the bST-63 groups compared to the bST-73 groups. Because no differences in pregnancy rates were observed between cows that received bST treatment at first injection of GnRH of the Ovsynch/TAI protocol (bST-63) or at timed insemination (bST-73), bST probably affects fertility responses after insemination. Such an interpretation agrees with data by Hernandez-Ceron et al. (2000), who reported increased pregnancy rates in cows with more than one service treated

with a single dose of bST at insemination. Therefore, the physiological window at which bST administration may enhance fertility appears to correspond to the period between ovulation and 32 d after insemination.

Such an effect could be mediated directly through increased plasma concentrations of bST or indirectly through increased concentrations of IGF-I produced by the liver in response to bST administration (Peel and Bauman, 1987). It has been observed that addition of growth hormone (i.e., bST) during maturation of cultured cumulus oocyte complexes accelerates nuclear maturation, induces cumulus expansion, and results in a subsequent increase in embryonic development (Izadyar et al., 1996). Further research indicated that growth hormone enhanced the developmental competence of the oocyte as a consequence of improved cytoplasmic maturation, which led to a higher fertilization rate (Izadyar et al., 1998). Such an effect is possible since the mRNA for the growth hormone receptor is expressed by bovine cumulus cells, mural granulosa cells, and by the oocyte (Izadyar et al., 1997). Moreover, addition of IGF-I to the in vitro maturation medium also stimulated nuclear maturation of the oocyte (Izadyar et al., 1997). Such an effect also may occur in vivo since IGF-I concentrations in follicular fluid were increased

after bST administration (Lucy et al., 1995). Therefore, bST administration may directly affect the pre-ovulatory follicle, affect oocyte maturation, and enhance fertilization to the Ovsynch/TAI protocol.

After fertilization, embryonic development may be influenced by bST and IGF-I, since both bST and IGF-I receptors have been identified in different stages of embryonic development (Palma et al., 1997; Simmen et al., 1995). Addition of IGF-I to cultured bovine embryos stimulated development in vitro (Kaye et al., 1992; Palma et al., 1997; Rieger et al., 1998). As discussed in Chapter 4, administration of bST at insemination in superovulated cows decreased the number of unfertilized oocytes, increased the percentage of transferable embryos, and increased the number of embryos in the blastocyst stage after embryo recovery 7 d after insemination.

It is possible that enhanced embryonic development may be modulated through differential oviductal function after bST administration because IGF-I receptors were identified in the bovine oviduct and uterine epithelium (Simmen et al., 1995) and addition of IGF-I stimulated the growth of cultured oviductal cells (Tiemann and Hansen, 1995). Moreover, plasma P_4 concentrations after insemination may affect embryonic development (Butler et al., 1996; Thatcher

et al., 1994). Treatment with bST may act through its receptor in luteal cells (Lucy et al., 1993) to increase CL weight (Lucy et al., 1995) and produce increased plasma P_4 concentrations in peripheral blood (Gallo and Block, 1991; Lucy et al., 1994; Schemm et al., 1990). However, analyses of plasma P_4 concentrations of a single sample collected at 6 d after insemination in a large number of cows in the present study failed to detect differences between cows receiving bST treatment and controls. Indeed, plasma P_4 concentrations of blood samples collected at 6 d after insemination also were not associated with pregnancy rates at 32 d after insemination. Such a result was not expected since it has been reported that a greater rise in plasma P_4 concentrations after insemination was associated with increased pregnancy rates (Butler et al., 1996; Thatcher et al., 1994). Perhaps collection of a single sample for P_4 determination after insemination was not enough to observe differences between cows that would be subsequently diagnosed pregnant or nonpregnant to the synchronized service. Nonetheless, present data do not support the hypothesis that pregnancy rates are associated positively with plasma P_4 concentrations after insemination and that

bST increases pregnancy rates due to its effects on plasma P_4 concentrations.

Administration of bST may attenuate the production of $PGF_{2\alpha}$ by the uterine endometrium at the time of maternal recognition of pregnancy and, thus, increase embryo survival. Growth factors such as IGF-I have been implicated in the regulation of phospholipase A_2 and cyclooxygenase-2 enzymes that regulate the endometrial synthesis of $PGF_{2\alpha}$ (Berenbaum et al., 1994; Jacques et al., 1997; Pruzanski et al., 1998). Addition of growth hormone to a bovine endometrial cell culture inhibited the expression of cyclooxygenase-2 and the secretion of $PGF_{2\alpha}$ (Badinga et al., 2000).

Administration of bST in the present study increased pregnancy rates in cyclic cows but not in anestrous cows. In a previous report, bST increased first-service pregnancy rates in a group of cows that potentially included both cyclic and anestrous cows (Moreira et al., 2000d). In the latter study, cows were only included in the experiment if their BCS was equal to or greater than 2.5 and this may have reduced the frequency of cows in anestrus at initiation of the Ovsynch/TAI protocol. As observed in the present study, frequency of cows in anestrus was negatively associated with BCS and cows with low BCS had significantly

higher frequency of anestrous. Thus, lower pregnancy rates for anestrous cows in the present experiment agree with previous observations that pregnancy rates to the Ovsynch/TAI protocol were reduced in cows with low BCS (Moreira et al., 2000c). When anestrous cows are submitted to the Ovsynch/TAI protocol, subsequent pregnancy rates are reduced, and the chances for bST to potentially influence oocyte maturation and embryonic development also are reduced as a consequence. It is also possible that bST or IGF-I may not have the same effects on the ovary and reproductive tract when cows are anestrus and have not had prior exposure to P_4.

An effect of pre-synchronization also was detected after anestrous cows were excluded. Such a pre-synchronization effect also was present when cyclic cows were not under the effects of bST at first service. A pre-synchronization system based on the use of two $PGF_{2\alpha}$ injections probably would not have an effect on anestrous cows since no estrus synchronization would be achieved. Pre-synchronization may have increased pregnancy rates to the first-service Ovsynch/TAI through three mechanisms: by enhancing health status of the reproductive tract; by increasing the number of estrous cycles before insemination; or by targeting cows to initiate the

Ovsynch/TAI protocol in a more favorable stage of the estrous cycle to enhance the percentage of cows with a synchronized ovulation.

Administration of $PGF_{2\alpha}$ constitutes an effective treatment for uterine infections and cystic ovaries (Chavatte et al., 1993; Steffan et al., 1984). Moreover, it has been postulated that increased number of estrous cycles before first service enhanced conception rates at first-service (Thatcher and Wilcox, 1973). Similarly, use of $PGF_{2\alpha}$ in postpartum cows has been reported to induce estrus before first service and increase first-service conception rates (Young et al., 1985). However, the prophylactic use of $PGF_{2\alpha}$ to increase herd reproductive performance has resulted in predominantly inconclusive results (Archbald et al., 1990; Armstrong et al., 1989; Etherington et al., 1994; McClary et al., 1989; Pankowski et al., 1995). Moreover, it is important to note that, in the present experiment, cows diagnosed with uterine disorders were excluded from the analyses and there were no indications that diagnosis of uterine infections occurred at a higher frequency in cows not pre-synchronized. Although pre-synchronization may enhance uterine health or increase the number of estrous cycles before the Ovsynch/TAI protocol, it seems improbable that these

effects would induce such a large difference in pregnancy rates as observed in the present experiment.

Alternatively, pre-synchronization affected the stage of the cycle at which the Ovsynch/TAI protocol was initiated. Interpretation from both original and alternative P_4-classes lead to the same conclusion. The frequency of cows classified as HIGH-LOW (or as high-low, according to alternative P_4-classes) was reduced in cows pre-synchronized. This category is probably comprised of cows receiving the first GnRH injection of the Ovsynch/TAI protocol at the late luteal phase of the estrous cycle, which may cause cows to be in estrus and ovulate before the time of insemination (Moreira et al., 2000b). Such an asynchrony between ovulation and insemination probably is responsible for the low pregnancy rates of HIGH-LOW cows observed previously (Moreira et al., 2000d) and in the present study. Therefore, reducing the frequency of cows classified as HIGH-LOW (and as high-low) during the Ovsynch/TAI protocol, as a consequence of pre-synchronization, enhanced first-service pregnancy rates.

Cows were less frequently classified as LOW-HIGH (or as low-high, according to alternative P_4-classes) for pre-synchronized groups compared with cows not pre-

synchronized. However, in contrast with previous results indicating that LOW-HIGH cows have reduced fertility after the Ovsynch/TAI protocol (Moreira et al., 2000d), LOW-HIGH cows in the present experiment had pregnancy rates similar to HIGH-HIGH cows. This difference between studies may reflect that, in the initial report (Moreira et al., 2000c), cows were not classified as anestrus or cyclic at the initiation of the Ovsynch/TAI protocol. It is likely that among LOW-HIGH cows, there were anestrous cows that responded to the initial GnRH injection of the Ovsynch/TAI protocol and had a lower pregnancy rate when compared to HIGH-HIGH cows (Moreira et al., 2000d).

As hypothesized, a greater percentage of cows were classified as HIGH-HIGH or as high-high after pre-synchronization compared with cows not pre-synchronized. Interestingly, pre-synchronization also increased pregnancy rates among cyclic cows classified as HIGH-HIGH or as high-high. Further analyses demonstrated that plasma P_4 concentrations of samples collected immediately before the first GnRH injection of the Ovsynch/TAI protocol did not differ, but plasma P_4 concentrations collected 7 d later, before injection of $PGF_{2\alpha}$, were greater for cows pre-synchronized than for cows not pre-synchronized. Moreover,

plasma P_4 concentrations at injection of $PGF_{2\alpha}$ were associated positively with pregnancy rates to the first-service Ovsynch/TAI. Increased plasma P_4 at injection of $PGF_{2\alpha}$ for pre-synchronized cows may be a consequence of the reduced variation in the stage of the cycle in which the Ovsynch/TAI protocol was initiated. The majority of pre-synchronized cows classified as HIGH-HIGH were expected to be between d 5 and d 11 of the cycle when the Ovsynch/TAI was initiated. In contrast, HIGH-HIGH cows in experimental groups not pre-synchronized may have initiated the Ovsynch/TAI protocol as early as d 3 of the cycle (when plasma P_4 concentrations may be greater than 1.0 ng/mL) or at the late luteal phase.

It is likely that more pre-synchronized HIGH-HIGH cows would ovulate to the first GnRH injection compared to HIGH-HIGH cows not pre-synchronized. Among pre-synchronized cows, a first-wave dominant follicle would most likely be present and responsive to an induced LH surge. In contrast, HIGH-HIGH cows which were not pre-synchronized may have ovulated at a reduced frequency since cows could have received the first GnRH injection before establishment of the first-wave dominant follicle (i.e., d 3 to d 4 of the cycle) or in the interval between the first and second

follicular waves (i.e., d 10 to d 15 of the cycle). Such a greater ovulation rate among pre-synchronized cows would explain the greater plasma P_4 concentrations observed by the latter group of cows compared with cows not pre-synchronized.

Cows that fail to ovulate to the first GnRH injection of the Ovsynch/TAI protocol are at greater risk for failure of synchronization of the emergence of a new follicular wave and have reduced conception to the synchronized service (Moreira et al., 2000b). Such an effect may provide further indication that, by increasing the percentage of cows ovulating to the first GnRH injection of the Ovsynch/TAI protocol and, thus, improving synchronization of follicular development, pre-synchronization may be subsequently increasing pregnancy rates to the timed insemination. This constitutes further evidence that pre-synchronization increased pregnancy rates to the first-service Ovsynch/TAI by targeting cows to initiate the synchronization program at the early luteal phase.

When cyclic HIGH-HIGH (or high-high) cows were re-categorized, it was observed that cows having intermediate plasma P_4 concentrations (i.e., between 1.0 and 2.5 ng/mL) at the first GnRH injection of the Ovsynch/TAI protocol had lower pregnancy rates compared to cows which had elevated

plasma P_4 concentrations (> 2.5 ng/mL). Pregnancy rates have been positively associated with plasma P_4 concentrations before insemination (Folman et al., 1990; Fonseca et al., 1983). This may explain why cows with intermediate plasma P_4 concentrations at the first GnRH injection of the Ovsynch/TAI protocol had lower pregnancy rates than cows with high plasma P_4 concentrations. Furthermore, cows with intermediate concentrations of plasma P_4 at the first GnRH injection probably initiated the Ovsynch/TAI protocol during the metestrus phase, a time when a dominant follicle may not have been established. Failure to ovulate a follicle after the first GnRH injection of the Ovsynch/TAI protocol may result in a failure of follicular synchronization and, therefore, reduce pregnancy rates to the timed insemination (Moreira et al., 2000b).

Previous reports indicated that cows having plasma P_4 concentrations greater than 1.0 ng/mL at 48 h after injection of $PGF_{2\alpha}$ had reduced pregnancy rates to the Ovsynch/TAI protocol (Moreira et al., 2000d). In the present study, when 1.0 ng/mL was used to distinguish between cows with complete or incomplete CL regression at 48 h after $PGF_{2\alpha}$, no differences in pregnancy rates were

observed. However, when the cut-off concentration of plasma P_4 for classifying cows as having complete or incomplete CL regression was set at 2.0 ng/mL, pregnancy rates were lower for cows with incomplete CL regression than for cows with complete CL regression. Collectively, these results indicate that failure of the injection of $PGF_{2\alpha}$ to induce complete CL regression during the Ovsynch/TAI protocol compromises subsequent fertility.

As expected, among cows classified as not ovulating after the second GnRH, according to both original and alternative P_4-classes, almost none conceived to the Ovsynch/TAI service. Among cyclic and anestrous cows classified as failing to ovulate to the second GnRH injection, only two cows were pregnant at 74 d after Ovsynch/TAI (3.4%, 2/58). These cows were borderline between being classified as ovulating to the second GnRH injection of the Ovsynch/TAI protocol. Such results indicate that classifying cows as failing to ovulate based on plasma P_4 may constitute a more practical diagnostic method for ovulation failure since it eliminates the need for ultrasonography examinations of the ovary, although it is not 100% accurate. According to results obtained for cyclic cows, 8.8% (or 9.2%, when alternative P_4-classes were

used for the analyses) of the cows failed to ovulate to the second injection of GnRH. This agrees with results from previous studies where ovulation failure was determined via ultrasonography (Pursley et al., 1995; Vasconcelos et al., 1999).

Although the limited number of cows classified as anestrous at the initiation of the Ovsynch/TAI protocol did not allow for extensive and sensitive comparisons among treatments, estimates of the effects of the protocol on such a group of cows were obtained. Pregnancy rates for cows that ovulated to both first and second GnRH injections were enhanced compared to pregnancy rates of cows that ovulated only after the second GnRH injection. Cows ovulating to both GnRH injections would have had a greater exposure to P_4 before insemination. The first estrous cycle after ovulation of anestrous beef cows had a reduced length, but due to prior uterine exposure to P_4, the second cycle was of normal length (Odde et al., 1980; Pratt et al., 1982). Present data indicate that pre-exposure to P_4 may be limited to a period of a few days (i.e., about 5 d of plasma P_4 greater than 1.0 ng/mL) to achieve acceptable pregnancy rates after insemination. Based on present results, pre-exposure to P_4 may be limited to a period of a

few days (i.e., about 5 d of plasma P_4 greater than 1.0 ng/mL) to achieve acceptable pregnancy rates after a timed insemination in anestrous cows. Furthermore, approximately 75% percent of anestrous cows ovulated to at least one of the two GnRH injections of the Ovsynch/TAI protocol. Since an ovulation rate of 90.5% occurred after the second GnRH (if anestrous cows also ovulated to the first GnRH) and pregnancy rates were as high as pregnancy rates of cyclic cows (39.6%), the Ovsynch/TAI protocol may stimulate anestrous cows to cycle earlier. The fact that pregnancy rates to the second service for anestrous cows were similar to those obtained for cyclic cows constitutes further evidence for a beneficial effect of the Ovsynch/TAI protocol.

Second-service pregnancy rates did not differ among treatment groups. An effect of pre-synchronization on second-service pregnancy rates was not expected because stage of the cycle at initiation of the second-service Ovsynch/TAI protocol was not influenced in cows pre-synchronized versus cows not pre-synchronized. The reason for the absence of an effect of bST treatment at second service is not clear. Overall, pregnancy rates to the second service were lower than pregnancy rates to the first service. This may be due to the fact that the Ovsynch/TAI

protocol for second service was initiated under heat stress conditions, as indicated by an effect of month of synchronization at the analysis of second-service Ovsynch/TAI. It also is possible that the relative frequency of sub-fertile cows increased at second compared to first service. Since overall fertility to the second-service Ovsynch/TAI was lower, and since bST effects on pregnancy rates appears to occur after first insemination, it would be more difficult to observe a beneficial effect of bST on pregnancy rates at later services. Alternatively, bST effects on the reproductive system may be differentially expressed after long-term treatment (i.e., at second service, cows were under bST influence for either 42 or 52 d) or during later stages of lactation, at which cows probably had reached a positive energy balance. Further investigation is necessary to clarify the effects of bST on fertility to second and subsequent services. However, no reduction in second or subsequent services due to bST treatment has been observed.

Because no treatment effects were observed at second service, differences in pregnancy rates at 115 d PP were due to differences detected previously on first service. Results demonstrate that pregnancy rates after two consecutive Ovsynch/TAI were greater for cows pre-

synchronized that received bST treatment compared to pre-synchronized cows not treated with bST. Such an effect of bST was not observed when cows were not pre-synchronized. That is probably because the magnitude of the bST effect on first-service pregnancy rates was less when cows were not pre-synchronized. Previous research also failed to detect bST effects on pregnancy rates at 120 and at 305 d PP (Moreira et al., 2000d).

As a consequence of results obtained at the first synchronized service, cows classified as anestrus at initiation of the first Ovsynch/TAI protocol had reduced pregnancy rates at 115 d PP. Thus, it is important to recognize that a delayed return to a cyclic status has long-term effects on herd fertility. A reduction in the frequency of cows in anestrus at the second month of lactation would have significantly enhanced treatment effects at first service and resulted in an improved overall herd performance at 115 d PP. This anestrus effect is even more prominent in primiparous cows. Further research is needed to identify risk factors which may increase the frequency of anestrous cows at the beginning of the breeding period and allow for better management of lactating cows before initiation of breeding programs.

No differences due to treatments were observed on the percentage of pregnancy losses between d 32 to 74 of pregnancy or after a positive pregnancy diagnosis at 74 d after insemination. Therefore, neither bST nor pre-synchronization treatments had deleterious effects on embryonic losses. Interestingly, a trend ($P < 0.10$) was observed indicating that pre-synchronized cows may have lower pregnancy losses between 32 and 74 d of pregnancy. The reason for such an effect is not clear at this time. Perhaps, quality of the ovulated oocyte can have long-term effects on embryonic losses after the period of maternal recognition of pregnancy. If that is true, it may be possible that, by increasing the percentage of cows that successfully synchronize their follicular development at initiation of the Ovsynch/TAI protocol, pre-synchronization is improving the quality of the ovulated oocyte and, therefore, increasing embryonic survival during the postimplantation period.

Neither pre-synchronization nor bST treatments affected the percentage of cows that required assistance at parturition. Hence, it may be deduced that treatments probably did not affect calf weight at parturition. Such an effect is in agreement with previous observations that

indicated no effects of bST on calf birth weight (Gallo and Block, 1990; Oldenbroek et al., 1993).

CONCLUSIONS

Both pre-synchronization treatment and bST administration successfully increased pregnancy rates to the first-service Ovsynch/TAI protocol. There were no indications that bST may reduce fertility of lactating cows submitted to a Ovsynch/TAI protocol that eliminates estrous detection. Administration of bST at initiation of the Ovsynch/TAI protocol or at insemination were equally beneficial to pregnancy rates, which indicates that bST possibly increases fertility in lactating dairy cows through its effects on oocyte maturation, embryonic development, and/or altered oviduct/uterine functions. Enhanced pregnancy rates in cows pre-synchronized were observed because cows were targeted to initiate the Ovsynch/TAI protocol at the early diestrous phase of the estrous cycle. Other pre-synchronization systems may be used for the same purpose, as long as a greater frequency of cows are targeted to initiate the Ovsynch/TAI protocol during the early luteal phase. Incidence of anestrus greatly influenced overall pregnancy rates. Further research is needed to improve the management of lactating

cows to decrease the frequency of anestrous cows at the second month of lactation.

CHAPTER 4
EFFECT OF BOVINE SOMATOTROPIN ON EARLY EMBRYONIC DEVELOPMENT AFTER SUPEROVULATION AND SUBSEQUENT PREGNANCY RATES OF LACTATING RECIPIENT COWS

INTRODUCTION

The use of recombinant bovine somatotropin (bST) in lactating dairy cattle has been associated with decreased reproductive performance. Lactating dairy cows receiving bST had increased number of days open (Zhao et al., 1992) and increased number of days to first service (Morbeck et al., 1991). Such effects on fertility may be associated with estrous detection rates, which were reduced when cows were treated with bST (Cole et al., 1992; Kirby et al., 1997; Morbeck et al., 1991). Hence, the potential effects of bST on estrous behavior potentially could be eliminated if cows were to receive a timed artificial insemination (Ovsynch/TAI) protocol that eliminates the need for detection of estrus. In two experiments designed to test this hypothesis (Moreira et al, 2001; Moreira et al., 2000d), bST actually caused an increase in first-service pregnancy rates among cows submitted to the Ovsynch/TAI program, where estrous detection was not a factor. The

magnitude of the increase in pregnancy rates when bST treatment was initiated before or at the time of insemination (Moreira et al., 2001). Similarly, increased pregnancy rates were observed in cows having more than one service that received a single dose of bST at insemination (Hernandez-Ceron et al., 2000). Thus, bST may be increasing pregnancy rates due to its effects after artificial insemination. Perhaps, bST increases fertilization rates after ovulation or enhances early embryonic development.

Several reports indicate that bST improves the total number of oocytes and embryos and number of transferable embryos after different superovulation treatments (Gong et al., 1993; Gong et al., 1996; Herrier et al., 1994; Rieger et al., 1991). In these studies, bST administration initiation was concurrent with superovulation treatments and the effect observed was attributed to an increase in the number of follicles ovulating in bST-treated donors (Gong et al., 1996; Herrier et al., 1994). Thus, the effect of bST treatment on fertilization rates and early embryonic development is confounded with the effects of bST on follicular development before ovulation. Initiation of bST treatment at the time of insemination after superovulation of donor cows would provide a model to

determine whether bST affects fertilization rates and early embryonic development.

In other reports, bST was administered either during superovulation treatments or at insemination of donors (Herrier et al., 1994; Kuehner et al., 1993). Results indicated a beneficial effect of bST when treatment was initiated before insemination and no effect when bST treatment was given at insemination. In neither of the previous reports was the stage of development and quality of recovered embryos examined, even though results from in vitro experiments indicate that either bST or insulin-like growth factor-I (IGF-I) can enhance early embryonic development (Izadyar et al., 2000; Kaye et al., 1992; Palma et al., 1997).

The present experiment was designed to test the effects of bST treatment given at insemination of donor cows on subsequent development and quality of flushed embryos. An additional objective was to evaluate the survival after transfer of embryos from bST-treated donors versus embryos from control cows. Finally, effect of bST on recipient cows was examined because it would provide further insight as to the mechanisms by which bST can increase pregnancy rates.

MATERIALS AND METHODS

The experiment was conducted on a commercial dairy located in Sparr, Florida, which has been using superovulation and embryo transfer of registered Holstein cattle as a major part of its reproductive management system for approximately 13 years. Both donor and recipient cows were kept in free stall facilities and fed a total mixed ration formulated based on nutrient requirements determined by the National Research Council to meet requirements for maintenance and milk production. Farm manager administered treatments according to instructions from the investigators.

Superovulation and Embryo Recovery

Eight lactating and four non-lactating Holstein cows were used as donors. Superovulation of donor cows was performed between November 1998 and August 1999 in a total of ten replicates. The interval between two consecutive superovulation treatments was at least 30 d. In this manner, a group of 6 to 10 donor cows per replicate received the after superovulation treatment: at experimental d 0, donor cows were injected with 2.5 mg of estradiol-17β and 50 mg of progesterone (i.m.) and received two progestin implants (Norgestomet; Synchro-Mate-B implant

only; Merial, Iselin, NJ; 6.0 mg, s.c.). On the afternoon of d 4 and then twice daily until the morning of d 8, donor cows received a sequence of eight decreasing doses of follicle-stimulating hormone (FSH; Folltropin-V,Vetrepharm, Bellevile, Canada; 20 mg/mL NIH FSH-P1; i.m.): two doses each of 4, 3, 2, and 1 ml. On the afternoon of d 6 and morning of d 7, donor cows received injections of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse, Pharmacia, Kalamazoo, MI; 25 mg; i.m.). Progestin implants were removed in the afternoon of d 7. After d 8, cows were observed for estrous behavior and inseminated at onset of estrus and every 12-h while estrus was expressed. Semen from twelve different sires was used at random to inseminate donor cows.

Cows not observed in estrus after superovulation treatment were excluded from the experiment. Treatments were initiated when donor cows received their first insemination. Donor cows were assigned randomly to a non-treated control group or to receive a single injection of bST (Posilac, Monsanto, St. Louis, MO; 500 mg; s.c.). Within each replicate, which included six to ten superovulated donor cows, treatments were assigned so that half of the experimental cows received bST treatment and the other half served as controls. Farm management made

the choice of donors and the decision relative to the number of times each cow received the superovulation protocol based upon the genetic merit of each donor and commercial needs of the dairy. After initial treatment assignment, treatments were alternated subsequently for every experimental donor, and, therefore, each donor served as experimental units for both control and bST treatments. Attention was given to the interval between consecutive superovulation treatments, which was required to be a minimum of 30 d. Donor cows had to be superovulated and flushed a minimum of two times to be included in the experiment and ten was the maximum number of times one single donor cow was flushed. Donor cows received one to four inseminations before embryo flushing. Only one cow received a single insemination whereas three cows received four inseminations.

At approximately 7 d after first insemination (range = 6.5 to 8.0 d), embryos were non-surgically recovered from donor cows (Ambrose et al., 1999). Embryos were then classified according to their stage of development and quality by one of the investigators who was unaware of donor treatments. Classification of embryos followed the standards adopted by the International Embryo Transfer Society (Stringfellow and Seidel, 1998). Embryos

determined to be in stages 2 or 3 of development (2- to 12-cell and early morula, respectively) and embryos whose quality was 3 or 4 (poor and dead/degenerating, respectively) were designated collectively as degenerate embryos. Degenerate embryos and oocytes recovered in stage 1 of development (i.e., unfertilized) were not frozen or transferred to recipient cows. Embryos determined to be in stages 4 to 7 of development (morula, early blastocyst, blastocyst, and expanded blastocyst, respectively) and with a 1 or 2 quality (excellent/good and fair, respectively) were designated as transferable embryos and frozen to be transferred into recipient cows.

Transferable embryos were placed into a freezing media containing 1.5 M ethylene glycol and 0.1 M sucrose (AB Technology; Pullman, WA) and loaded into 0.25 cc straws for a total exposure time of 8 min in the cryoprotectant solution before seeding. Ice crystal formation was initiated outside the cryochamber by touching the straws with a cotton ball dipped in liquid nitrogen on the top and bottom. Straws were then immediately placed in the cryochamber (Biogenics Inc., Napa, CA) and kept at - 6 °C for 10 minutes. The freezer was then programmed to decrease temperatures at a rate of 0.6 °C per minute to a final temperature of - 32 °C. Straws were then transferred

to and stored in a tank containing liquid nitrogen until thawed for transfer.

Recipient Management and Embryo Transfer

Lactating Holstein primiparous and multiparous cows (n = 181) were used as recipients. Recipient cows were detected in estrus by visual observation conducted throughout the day by farm personnel. At approximately 12 h after estrus was detected (i.e., correspondent insemination time), recipient cows were assigned randomly to initiate bST treatment (Posilac; 500 mg; s.c.) at this time or to serve as untreated controls. Once bST treatment was initiated, recipient cows were re-injected with bST every 14 d until approximately 30 d before dry off. At approximately 7 d after estrus (range = 6 to 7 d), recipient cows received frozen/thawed experimental embryos, which were transferred non-surgically. Embryos to be transferred to recipients were chosen at random within treatment (i.e., bST or control) and assigned randomly to the available group of recipient cows at the beginning of each year in an attempt to keep the number of experimental units similar among treatment groups. Therefore, four groups in a 2x2 factorial experiment were formed: control recipients that received embryos from control donors

(control-recipient/control-embryo; n = 43), control recipients that received embryos from bST-treated donors (control-recipient/bST-embryo; n = 41), bST recipients transferred with control embryos (bST-recipient/control-embryo; n = 37), and bST recipients that were transferred with embryos from bST-treated donors (bST-recipient/bST-embryo; n = 60). At approximately 40 to 50 d after estrus (i.e., 33 to 43 d after embryo transfer), cows were examined for pregnancy by rectal palpation. After a positive diagnosis for pregnancy, records of experimental cows were followed up until calving. The number of cows that lost their pregnancy and calving difficulty scores (1 = no intervention required, 2 = slight intervention required, 3 = moderate intervention required, 4 = heavy intervention required, 5 = caesarean section required) were recorded for subsequent analyses.

Initially, only first service recipient cows were assigned to treatment groups (n = 163). However, in order to increase the number of observations within the experiment, recipient cows in the control treatment (i.e., cows not treated with bST) that did not conceive after first service embryo transfer were re-assigned randomly to either bST or control treatments for their second service (n = 18). Recipient cows assigned to control group did not

receive bST treatment until after pregnancy diagnosis was performed. After pregnancy diagnosis, control recipients initiated bST treatment according to farm management discretion. Experimental recipient cows were between their first and eighth lactation and were divided into primiparous (n = 83) and multiparous cows (n = 93). Recipient cows received embryo transfer as early as 37 d postpartum and as late as 332 d postpartum. Less than 5% of recipient cows were greater than 180 d postpartum at embryo transfer (n = 9). The median value for the number of d postpartum at embryo transfer was 97 d. Thus, recipient cows were divided in two groups according to stage of lactation at embryo transfer: cows that were less than or equal to 97 d postpartum (n = 94) or cows that were greater than 97 d postpartum at embryo transfer (n = 87). Farm manager (technician 1; n = 133) and one of the investigators (technician 2; n = 48) performed embryo transfer. Recipient cows were transferred with experimental embryos from January until September of 1999 (first year; n = 135). Embryo transfer was discontinued for the remaining of 1999 and re-initiated from January until March 2000 (second year; n = 46). Embryos transferred between the months of January and March were designated as cool season transfers (n = 140), whereas

embryos transfers occurring between April and September were designated as hot season transfers (n = 41).

Statistical Analyses

Experimental responses from embryo flushes were performed using the method of least squares ANOVA of the General Linear Model (GLM) procedure of the Statistical Analysis System software package (SAS, 1988). Statistical models included the effects of donor treatment, milking status (i.e., lactating versus non-lactating cows), cow nested within milking status, flushing replicate, season (cool versus hot season), number of inseminations at superovulated-induced estrus before flushing (i.e., $\leq$ 2 inseminations versus > 2 inseminations), sire and interval from first insemination to embryo flushing as a continuous variable, as well as higher order interactions. The effects of flushing replicate, season, number of inseminations before flushing, and interval from first insemination to flushing as well as higher order interactions were not significant and, therefore, were removed from the final model which included the effects of treatment, milking status and cows nested within milking status. The percentage of transferable embryos relative to the total number of oocytes and embryos recovered, and the

frequency distributions of transferable embryos classified according to stage of development and quality were analyzed by chi-square analyses.

The GLM, Genmod, and Logistic procedures of SAS (SAS, 1988) were used to analyze pregnancy rates of recipient cows after embryo transfer as well as frequency of abortions and difficulty scores at calving. Since analytical responses were similar between the three different procedures, logistic regression analyses were used to report results. The mathematical model included the effects of donor treatment, recipient treatment, interaction between donor and recipient treatments, stage of development (i.e., embryos in morula stage versus embryos in early blastocysts, blastocyst, and expanded blastocyst stage of development), quality of embryos transferred, milking status of embryo donor (i.e., embryos from lactating or non-lactating donors), year and season of transfer, parity, stage of lactation and service number of recipient cows, technician performing embryo transfer, interval from estrus to transfer (i.e., 6 or 7 d) and higher order interactions. Only variables significant at the 0.20 level were maintained in a reduced model, which included the effects of donor and recipient treatments, the interaction between donor and recipient treatments and

quality of embryo transferred on pregnancy rates of recipient cows. Differences were considered significant at $P < 0.05$.

RESULTS

Embryo Flushing

Results from a total of 52 embryo flushes (26 from control-treated cows and 26 from bST-treated donor cows) were used for the analyses. Superovulation of donor cows and embryo recovery were performed once every 30 d from November 1998 to August 1999 in a total of 10 replicates. Every cow was flushed at least two times and one donor cow was flushed in every replicate (i.e., ten times). Recovery results from November 1998 until March 1999 were considered to have occurred during the cool season (n = 26 flushes; 13 from control-treated and 13 from bST-treated donors) and recovery results from flushes that took place from April 1999 to August 1999 were considered to have occurred during the hot season (n = 26 flushes; 13 from control-treated and 13 from bST-treated donors). Results from 30 flushes were from non-lactating cows (15 flushes from control-treated and 15 flushes from bST-treated donors) and results from 22 flushes were from lactating cows (11 flushes from control-treated and 11 flushes from bST-treated donors). Donor

cows were inseminated one to four times. Only one bST-treated donor was inseminated once and three donor cows were inseminated a total of four times (2 bST-treated and 1 control-treated donor), and cows were divided into a group that received $\leq$ 2 inseminations (n = 22 flushes) and into a second group that received > 2 inseminations (n = 30 flushes). A total of 51 flushes occurred within 7 d ± 12 h from the first insemination (26 flushes from control-treated and 25 flushes from bST-treated donors). Only one flush from a bST-treated donor occurred 8 d after first insemination. Number of total recovered oocytes and embryos, unfertilized ova, degenerate embryos, transferable embryos per flush and stage of embryonic development were not affected by number of flushes, season, number of inseminations, or interval between first insemination and embryo recovery.

Number of unfertilized ova was reduced in bST-treated donor cows as compared to control donors ($P < 0.04$; Table 4.1). The percentage of embryos classified as transferable relative to the total number of oocytes and embryos recovered was increased ($P < 0.01$) for bST-treated donors (77.2%, 206/267) as compared to control-treated donors (56.4%, 150/266). Analyses of the number of transferable embryos per flush according to stage of embryonic

development indicated that the numbers of embryos classified as morula, early blastocyst and expanded blastocyst were not affected by treatment, but the number of embryos per flush classified as blastocyst was increased ($P < 0.04$) in bST-treated donors as compared to control (Table 4.1).

Table 4.1. Total recovered oocytes/embryos, unfertilized ova, degenerate embryos, transferable embryos, and number of embryos according to stage of development and quality per flush for bST and control-treated donor cows.

Variable	Control donors (embryos/flush)	bST donors (embryos/flush)
Total oocytes/embryos	9.4 ± 1.5	9.3 ± 1.4
Unfertilized oocytes	3.7 ± 0.9 [a]	1.0 ± 0.9 [b]
Degenerate embryos	0.3 ± 0.3	0.9 ± 0.3
Transferable embryos	5.4 ± 1.4	7.4 ± 1.4
Stage of development:		
- Morula	1.7 ± 0.4	1.5 ± 0.4
- Early blastocyst	2.9 ± 0.8	3.0 ± 0.8
- Blastocyst	0.4 ± 0.6 [a]	2.4 ± 0.6 [b]
- Expanded blastocyst	0.4 ± 0.4	0.5 ± 0.4
Embryo quality:		
- Excellent/good	4.9 ± 1.2	6.2 ± 1.2
- Fair	0.9 ± 0.3	1.2 ± 0.3

[a,b] Superscripts within row indicate difference ($P < 0.04$).

Differences were observed when the frequency distribution of transferable embryos classified according to stage of development was analyzed across treatments ($P < 0.01$; Figure 4.1). The frequency distribution of embryos classified as excellent/good or as fair did not differ between bST (82.5% [170/206] and 17.5% [36/206], respectively) and control donors (81.3% [122/150] and 18.7% [28/150], respectively).

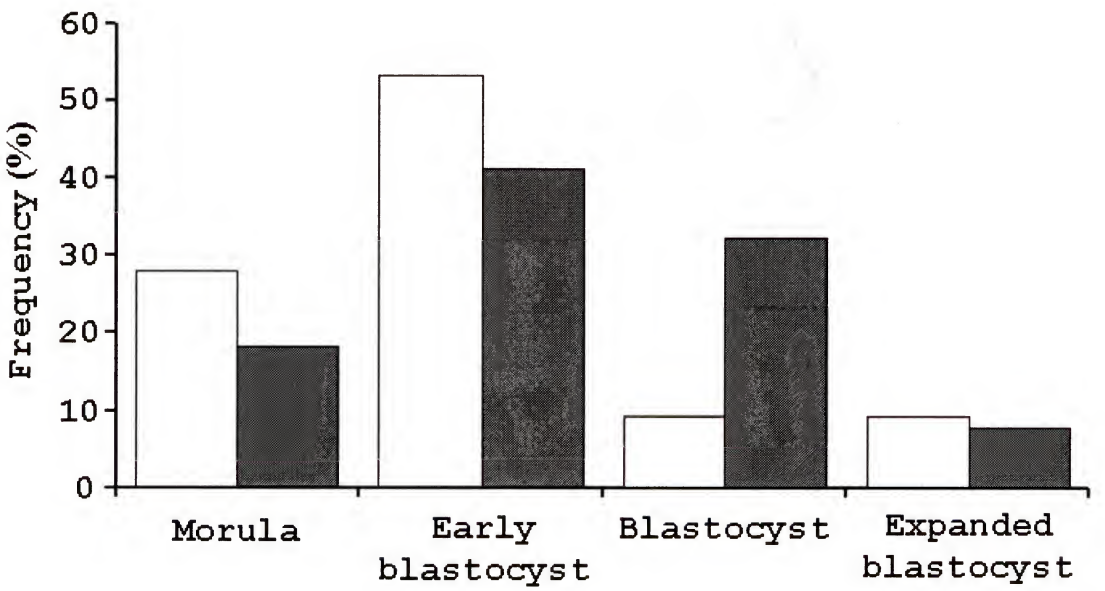

Figure 4.1. Frequency distribution of transferable embryos across stages of development for control (white bars) and bST treatments (gray bars). A difference in the frequency distribution across stages of embryonic development was detected ($P < 0.01$).

Results also were affected by milking status of donor cows. Non-lactating cows had a greater ($P < 0.01$) number of total recovered oocytes and embryos (Table 4.2). Although the number of recovered embryos classified as degenerate per flush was not affected by milking status, the number of recovered unfertilized ova per flush was greater in non-lactating cows than in lactating cows ($P < 0.04$; Table 4.2). As a consequence, the percentage of embryos classified as transferable relative to the total number of oocytes and embryos recovered was increased ($P < 0.05$) for lactating donors (75.2%, 91/121) as compared to non-lactating donors (64.3%, 265/412). Nonetheless, the number of transferable embryos per flush for non-lactating cows was increased ($P < 0.04$) as compared to lactating cows (Table 4.2). The number of transferable embryos per flush classified as morula was increased in non-lactating cows ($P < 0.04$), but the number of early blastocysts, blastocysts, and expanded blastocysts were not affected by milking status (Table 4.2). Frequency of transferable embryos classified according to stage of development did not differ between lactating and non-lactating donors (Figure 4.2). More embryos of fair quality were recovered from non-lactating cows than from lactating cows ($P < 0.05$), and a trend ($P < 0.07$) was observed for an increase in the number

of excellent quality embryos in non-lactating cows as compared to lactating cows. However, the frequency distribution of embryos classified as excellent/good or as fair did not change between lactating (85.7% [78/91] and 14.3% [13/91], respectively) and non-lactating donors (80.8% [214/265] and 19.2% [51/265], respectively).

Table 4.2. Number of total recovered oocytes/embryos, unfertilized ova, degenerate embryos, transferable embryos, and number of embryos according to stage of development and quality per flush for lactating and non-lactating donor cows (LSM ± SE).

Variable	Lactating (embryos/flush)	Non-lactating (embryos/flush)
Total oocytes/embryos	5.5 ± 2.4 [a]	13.3 ± 2.0 [b]
Unfertilized oocytes	0.9 ± 0.6 [c]	3.9 ± 0.5 [d]
Degenerate embryos	0.4 ± 0.3	0.8 ± 0.2
Transferable embryos	4.2 ± 2.1 [c]	8.6 ± 1.8 [d]
Stage of development:		
- Morula	0.8 ± 0.7 [c]	2.4 ± 0.6 [d]
- Early blastocyst	2.2 ± 1.4	3.7 ± 1.1
- Blastocyst	0.9 ± 1.0	1.9 ± 0.9
- Expanded blastocyst	0.3 ± 0.5	0.6 ± 0.4
Embryo quality:		
- Excellent	3.6 ± 1.8	7.1 ± 1.5
- Good	0.6 ± 0.5 [e]	1.5 ± 0.4 [f]

[a,b] Superscripts within row indicate differences ($P < 0.01$).
[c,d] Superscripts within row indicate differences ($P < 0.04$).
[e,f] Superscripts within row indicate differences ($P < 0.05$).

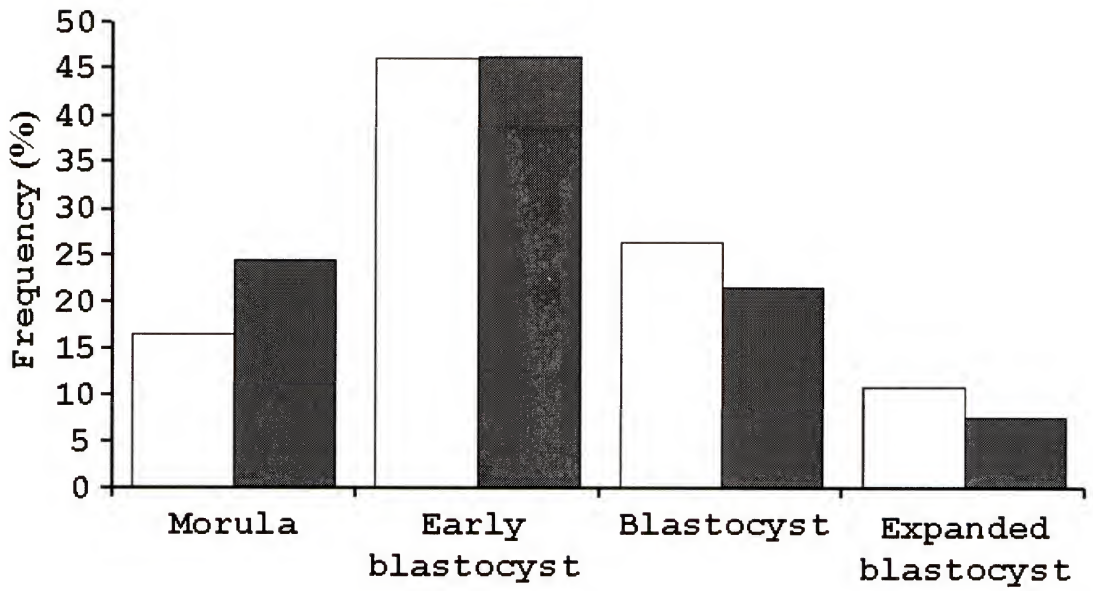

Figure 4.2. Frequency distribution of transferable embryos across stages of development for lactating (white bars) and non-lactating donors (gray bars). No differences in the frequency distribution across stages of embryonic development were detected.

Embryo Transfer

Results from the complete multivariable logistic regression model are depicted in Table 4.3. Only variables significant at the 20% level were maintained in the subsequent reduced logistic regression model, which is depicted in Table 4.4. The effect of recipient treatment was included in the reduced logistic regression model because a trend ($P < 0.10$) for an interaction between

recipient and embryo treatments was detected. Figure 4.3 depicts a graphic representation of the interaction between embryo and recipient treatments. Overall pregnancy rate to embryo transfer was 42.0% (76/181).

Table 4.3. Pregnancy rates for variables in the complete logistic regression model.

Factor	Pregnancy rates (pregnant/total cows)		P value
Embryo treatment	Control	bST	
	33.7% (27/80)	48.5% (49/101)	< 0.03
Recipient treatment	Control	bST	
	40.5% (34/84)	43.3% (42/97)	0.94
Interaction	Group 1 [1]	Group 2 [2]	
	35.9% (37/103)	50.0% (39/78)	< 0.08
Embryo quality	Excellent	Good	
	47.4% (73/154)	11.1% (3/27)	< 0.01
Stage of development	Morula	Blast [3]	
	25.6% (10/39)	46.5% (66/142)	0.25
Year	First	Second	
	43.7% (59/135)	37.0% (17/46)	0.24

Table 4.3. Continued.

Factor	Pregnancy rates (pregnant/total cows)		P value
Season	Cool	Hot	
	44.3% (62/140)	34.2% (14/41)	0.25
Parity	Primiparous	Multiparous	
	43.2% (38/88)	40.9% (38/93)	0.67
Stage of lactation	≤ 97 d PP	> 97 d PP	
	45.7% (43/94)	37.9% (33/87)	0.37
Transfer number	First	Second	
	42.9% (70/163)	33.3% (6/18)	0.50
Technician	Technician 1	Technician 2	
	42.9% (57/133)	39.6% (19/48)	0.67
Estrus to transfer [4]	6 days	7 days	
	37.9% (11/29)	42.2% (65/152)	0.49
Donor Status	Non-lactating	Lactating	
	42.4% (53/125)	41.0% (23/56)	0.33

[1] Pooled data from control-recipient/control-donor and bST-recipient/bST donor.
[2] Pooled data from control-recipient/bST donor and bST-recipient/control-donor.
[3] Blastocysts consist of embryos classified as in the early blastocyst, blastocyst, and expended blastocyst stage.
[4] Interval from first estrus to embryo transfer.

Table 4.4. Pregnancy rates, odd ratios (OR) and 95% confidence interval (CI) for risk of conception.

Factor	Adjusted odd ratios	95% confidence interval	P value
Recipient treatment			0.79
- Control	1.0		
- bST	1.1	0.6 - 2.1	
Embryo treatment			0.03
- Control	1.0		
- bST	2.0	1.1 - 3.9	
Interaction			0.05
- Group 1[1]	1.0		
- Group 2[2]	1.9	1.0 - 3.6	
Embryo quality			< 0.01
- Good	1.0		
- Excellent	7.4	2.1 - 26.2	

[1] Pooled data from control-recipient/control-donor and bST-recipient/bST donor.
[2] Pooled data from control-recipient/bST donor and bST-recipient/control-donor.

Due to the presence of an interaction between embryo and recipient treatments, pregnancy data were further examined. Because there were no differences in pregnancy rates between recipient cows treated with bST, regardless of whether they received a control or bST embryo, data from these two groups were pooled (bST-recipient) and compared alternatively to pregnancy rates from the control-

recipient/control-embryo group and to the control-recipient/bST-embryo group. The probability of conception was greater for the pooled bST-recipient group than for the control-recipient/control-embryo group (adjusted odd ratio = 2.2; 95% confidence interval = 1.02 to 5.08; $P < 0.05$). However, there were no differences in the probability of conception between the pooled bST-recipient group and the control-recipient/bST-embryo group (adjusted odd ratio = 0.6; 95% confidence interval = 0.28 to 1.24; $P = 0.17$).

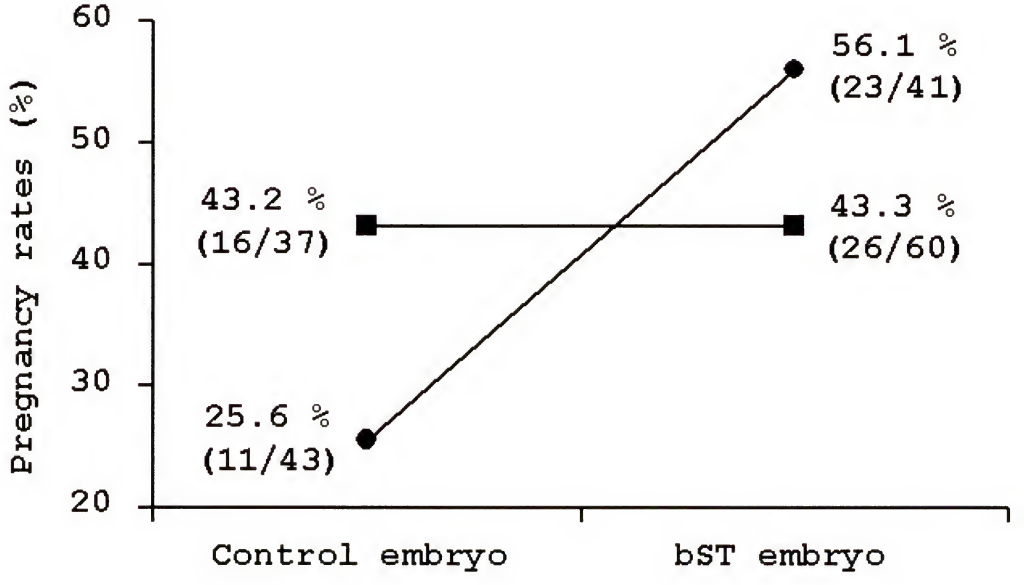

Figure 4.3. Pregnancy rates after embryo transfer depicting the interaction ($P < 0.05$) between embryo and recipient (closed circle = control treatment; closed square = bST treatment) treatments.

Among the overall number of pregnant recipient cows (n = 76), two recipients died before calving and therefore were excluded from the analyses of frequency of abortions and calving difficulty. Both recipients were from the control group that had received control embryos. Overall, 12.2% (9/74) of recipient cows diagnosed pregnant 40 to 50 d after detection of estrus, lost their pregnancy before calving. The incidence of abortions was similar ($P > 0.6$) between recipients transferred with bST or control treated embryos (10.2% [5/49] and 16.0% [4/25], respectively) and between recipients that receiving bST or control treatments (11.9% [5/42] and 12.5% [4/32], respectively). No interaction between donor and recipient treatments was observed in these analyses ($P > 0.6$). Moreover, the incidence of abortions was not affected by any of the remaining variables in the statistical model ($P > 0.3$).

Aborted recipient cows were excluded from calving difficulty analyses. The percentage of recipient cows receiving bST-treated embryos that needed assistance at calving was 27.3% (12/44) which did not differ ($P > 0.2$) from the percentage of recipient cows receiving a control embryo that needed intervention at parturition (14.3%, 3/21). Similarly, there was no difference ($P > 0.8$) between the percentage of bST-treated recipient cows that

needed assistance at parturition as compared to control-recipient cows (24.3% [9/37] and 21.4% [6/28]. No interaction between embryo and recipient treatments was observed (P > 0.8). Moreover, calving difficulty (i.e., no intervention versus intervention at calving) was not affected by any of the remaining variables included in the statistical model (P > 0.2) with the exception of parity. Thus, calving difficulty was re-analyzed in a model that included only the effect of parity. Results from such an analysis indicated that the incidence of cows with some degree of calving difficulty was not affected by parity (primiparous = 30.3% [10/33] and multiparous = 15.6% [5/32]; P = 0.16).

DISCUSSION

The present experiment was designed to characterize embryonic and maternal mechanisms by which exogenous bST affects fertility responses in lactating dairy cows subjected to the Ovsynch/TAI protocol as observed previously (Moreira et al., 2001; Moreira et al., 2000d). Results support the hypothesis that bST may improve fertility of dairy cows and that bST influences reproductive events after insemination.

Administration of bST at the time of insemination of donor cows did not affect number of total recovered oocytes and embryos, indicating that bST treatment did not affect number of follicles ovulating after superovulation. Injection of bST as part of the superovulation protocol increased the number of follicles ovulating after superovulation (Gong et al., 1993; Gong et al., 1996; Rieger et al., 1991). This was attributed to an increase in the population of small antral follicles induced when bST was initiated concurrently with superovulation treatment (Gong et al., 1993; Gong et al., 1996). In the present study, however, bST was given at approximately 12 h before ovulation at the time of insemination.

Administration of bST at insemination decreased the number of unfertilized follicles recovered 7 d after insemination. There were no differences in the number of transferable embryos per flush, even though administration of bST produced an average of two more embryos per flush (Table 4.1). This was attributed to the variability in number of embryos flushed among different donors. Because of the lower frequency of unfertilized embryos, the percentage of embryos classified as transferable relative to the total number of recovered oocytes and embryos was increased when cows received bST. Hence, bST treatment

increased fertilization rates of donor cows. This could be attributed to a direct effect of bST or an indirect effect, mediated via increased plasma concentrations of IGF-I (Lucy et al., 1995). Therefore, bST or IGF-I either may increase the fertilization competence of the sperm or enhance the capacity of the oocyte to become fertilized.

Cumulus cells, mural granulosa cells and oocytes express mRNA for the bST receptor (Izadyar et al., 1997b, Kolle et al., 1998). Treatment with bST stimulates cumulus expansion and nuclear maturation of oocytes and increases fertilization, based on increased cleavage rates of oocytes matured in the presence of bST (Izadyar et al., 1996; Izadyar et al., 1998a; Izadyar et al., 1998b). These effects on oocyte maturation were not mediated by IGF-I because addition of antibody to IGF-I did not inhibit the stimulatory effect of bST (Izadyar et al., 1997b). Therefore, in vitro data support the hypothesis that bST administration at insemination in donor cows may directly affect oocyte maturation and increase fertilization rates. Addition of both IGF-I and epidermal growth factor to the maturation medium of bovine oocytes stimulated progression of meiosis, as denoted by an acceleration in the timing of extrusion of the first polar body (Sakaguchi et al., 2000), and also stimulated responses such as cumulus expansion,

oxidative metabolism, nuclear maturation and cleavage rate after fertilization (Izadyar et al., 1997b; Lorenzo et al., 1994; Matsui et al., 1995; Rieger et al., 1998). In buffalos, cleavage rates are increased when oocytes are matured in the presence of IGF-I (Pawshe et al., 1998). Because bST treatment increases intra-follicular concentrations of IGF-I of the dominant follicle (Lucy et al., 1995) or in pre-ovulatory follicles after superovulation (Herrier et al., 1994), it is possible that the capacity of the oocyte to undergo fertilization is enhanced through an effect of IGF-I after administration of bST.

The observation that bST treatment increased fertilization rates of donor cows may also be related to the capacity of sperm to fertilize oocytes. Administration of somatotropin (ST) to ST-deficient rats and humans increased sperm concentration and motility (Breier et al., 1996; Homburg and Farhi, 1995) and the increase in motility coincided directly with increased seminal plasma IGF-I (Homburg and Farhi, 1995, Ovesen et al., 1996). Seminal plasma concentrations of IGF-I were correlated positively with sperm numbers in fertile and infertile men such that seminal plasma concentrations of IGF-I may serve as a marker of semen quality (Colombo and Naz, 1999). In

cattle, administration of bST to bulls increased sperm motility and fertility after freezing and thawing (Sauerwein et al., 2000), and serum concentrations of IGF-I were correlated with the percentage of normal sperm cells (Yilmaz et al., 1999). Messenger RNA for IGF-I and IGF-I receptor were identified in oviduct cells and uterine endometrium from cows (Geisert et al., 1991; Lonergan et al., 2000), and maximal concentrations of IGF-I in the uterine lumen of both sheep and cows occur at around estrus, during the time of sperm transport (Geisert et al., 1991; Ko et al., 1991).

Treatment with bST accelerated early embryonic development. The number of recovered embryos at the blastocyst stage was increased when donors were treated with bST as compared to controls. Furthermore, the frequency distribution of embryos among different stages of development was affected by bST treatment. A greater percentage of transferable embryos were in more advanced stages of development (i.e., blastocyst and expanded blastocyst) as compared to control donors. The acceleration of early embryonic development caused by bST is supported by in vitro experiments. Addition of ST to culture medium significantly increased the percentage of embryos that reached the blastocyst stage (Izadyar et al.,

2000). This is probably a direct effect of bST because bovine embryos as early as at the 2-cell stage of development express mRNA for ST receptor (Izadyar et al., 2000), and mRNA for ST (Lechniak et al., 1999). Enhanced early embryonic development was also observed when ST was added during culture of mouse embryos (Drakakis et al., 1995; Fukaya et al., 1998).

Furthermore, addition of IGF-I during bovine embryo culture enhanced early embryonic development (Kaye et al., 1992; Palma et al., 1997). Interestingly, it was observed that a shorter time interval from insemination to first cleavage in vitro (i.e., < 36 h) was associated with a greater rate of development to the blastocyst stage in cattle (Lonergan et al., 1999). Expression of IGF-I receptor mRNA was detected in 2-cell embryos that cleaved less than 36 h after insemination, whereas embryos that cleaved later than 36 h after insemination did not express mRNA for IGF-I (Lonergan et al., 2000). Thus, expression of IGF-I ligand may be linked to time of first cleavage and act as an indicator of embryonic developmental competence. Because IGF-I mRNA expression peaks around the ovulatory period in the cow oviduct (Schmidt et al., 1994), a model was proposed where IGF-I may be released into the oviductal fluid to directly stimulate embryo growth or,

alternatively, stimulate secretory activity of the ampulla (Wathes et al., 1998), whose activity also peaks around ovulation (Murray, 1996). Collectively, these findings suggest that bST administration at the time of insemination of donor cows enhanced early embryonic development by potentially increasing ST and IGF-I concentrations within oviductal and uterine tissues.

Lactation status significantly affected embryo recovery results. Total number of recovered oocytes and embryos was increased greatly for non-lactating cows as compared to lactating cows. However, the number of unfertilized ova also was increased. Despite the increase in non-fertilized oocytes and embryos, number of transferable embryos still remained greater for non-lactating cows, but the percentage of embryos classified as transferable relative to the total number of recovered oocytes and embryos was less for non-lactating cows than for lactating cows. The number of follicles ovulating in response to superovulation was greater in non-lactating than in lactating cows, but subsequent fertilization rates were greater in lactating cows. Regardless of fertilization rates, non-lactating cows had a clear advantage that the number of transferable embryos per flush was greater. Despite an increase in the number of

transferable embryos and in the number of recovered embryos at the morula stage for non-lactating cows, no differences in the frequency distribution of embryos among stages of development were detected between non-lactating and lactating cows. Similarly, even though the number of embryos of excellent/good or fair quality tended to increase or were increased in non-lactating cows, the percentage of embryos across quality classifications did not differ between non-lactating and lactating cows. Non-lactating cows had greater number of recovered embryos, but their developmental distribution and quality were equivalent to lactating cows on a percentage basis.

Transfer of bST-treated embryos increased pregnancy rates of recipient cows. The observed effect of bST treatment of donors on subsequent pregnancy rates was independent of quality and stage of development of transferred embryos because no interactions were observed. Thus, donor effect was not solely attributable to greater development to the blastocyst stage. The reason for such an effect is not clear. The ovine conceptus secreted a greater amount of interferon-τ (IFN-τ) in response to a combination of IGF-I and IGF-II (24). Perhaps, bST-treated embryos had an enhanced capacity to secrete IFN-τ that contributed to a higher rate of embryonic survival during

the period of maternal recognition of pregnancy. In humans, addition of IGF-I to in vitro derived embryos did not affect the total number of cells per embryo, but decreased the proportion of apoptotic cells (Spanos et al., 2000). In cattle, addition of IGF-I and IGF-II decreased the apoptotic cell index and increased total cell number of blastocyst s at d 7 of development (Byrne et al., 1999). Therefore, treatment of donors with bST may have resulted in embryos with a greater number of viable cells and, as a consequence, increased the capacity of the embryo to withstand the freezing process and survive in vivo after transfer to recipient cows.

Moreover, there was no interaction between treatments, stage of embryonic development and interval from insemination to embryo transfer. Because transfer of embryos in different stages of development needs to be synchronized with stage of the cycle at embryo transfer, it could be anticipated that an interaction would be present. Perhaps, the number of embryo transfers was not enough to detect such an interaction.

The interaction between donor and recipient treatments on pregnancy rates was interpreted to indicate that bST treatment of recipient cows increased pregnancy rate compared to control recipient cows that received a control-

treated embryo. However, pregnancy rates of recipient cows treated with bST did not differ from pregnancy rates of control recipient cows receiving a bST-treated embryo. Therefore, the positive effects of bST treatment on recipient and donor-produced embryos were not additive.

Reports in the literature provide potential mechanisms by which bST may have increased pregnancy rates. Receptors for both bST and IGF-I are present within the endometrium of cows (Lucy et al., 1998; Wathes et al., 1998). In the uterus of cyclic cows and sheep, IGF-I receptor mRNA is localized mainly in the epithelium of the endometrial glands (Stevenson et al., 1994; Wathes et al., 1998), and expression was highest in the mid luteal phase of cows (Wathes et al., 1998). The high concentration of IGF-I receptors on uterine epithelial glands suggests a role for IGF-I in regulating glandular secretory activity, that contributes to an intra-uterine environment capable of sustaining embryonic development in the preimplantation period when the embryo is dependent upon histotrophic nutrition (Wathes et al., 1998). Therefore, increasing circulating levels of both bST and IGF-I as a consequence of bST treatment may stimulate secretory activity of endometrial glands and enhance the uterine environment to sustain a pregnancy.

Furthermore, IGF-I and other growth factors were implicated in the regulation of phospholipase A_2 and cyclooxygenase-2 enzymes (Jacques et al., 1997; Pruzanski et al., 1998). Addition of GH to a bovine endometrial cell culture inhibited the expression of cyclooxygenase-2 and the secretion of $PGF_{2\alpha}$ in response to stimulation by a synthetic phorbol ester (Badinga et al., 2000). It is possible that bST treatment of recipient cows may attenuate the production of $PGF_{2\alpha}$ at the time of maternal recognition of pregnancy permitting greater embryonic survival.

Treatment of recipient cows with bST and transfer of bST-treated embryos did not affect frequency of abortions after a positive pregnancy diagnosis at 33 to 43 d after embryo transfer (i.e., 40 to 50 d after estrus). Treatment of donors or recipients with bST did not affect the percentage of cows that needed assistance at calving. This agrees with previous observations that bST did not increase calf birth weight (Gallo and Block, 1990; Oldenbroek et al., 1993).

CONCLUSIONS

Administration of bST at the time of insemination enhances subsequent pregnancy rates of lactating dairy cows. Both maternal and embryonic factors appear to

contribute to the improvement in pregnancy rate. Further research is warranted to elucidate the mechanisms in which bST administration increases pregnancy rate.

CHAPTER 5
EFFECTS OF GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-I ON DEVELOPMENT OF IN VITRO DERIVED BOVINE EMBRYOS

INTRODUCTION

Recent reports indicate that growth hormone (GH) and insulin-like growth factor-I (IGF-I) may affect oocyte maturation, early embryonic development, and pregnancy rates in cattle. Growth hormone receptor mRNA has been identified in cumulus cells, mural granulosa cells, and in the oocyte of bovine ovarian follicles (Izadyar et al., 1997b; Kolle et al., 1998). Furthermore, GH mRNA and protein are produced by granulosa, theca, cumulus cells and both immature and mature oocytes (Izadyar et al., 1999). Thus, GH may exert a paracrine or autocrine role during oocyte maturation. In addition, GH and IGF-I may influence early embryonic development because mRNA for GH and IGF-I receptors were identified in the developing embryo as early as in the one cell stage (Izadyar et al., 2000; Watson et al., 1992). Moreover, concentrations of IGF-I in the oviduct lumen are increased during the periovulatory period (Schmidt et al., 1994), and both GH and IGF-I mRNA are

expressed by bovine embryos (Lechniak et al., 1999; Lonergan et al., 2000).

Administration of recombinant bovine GH to lactating dairy cows increases milk production (Peel and Bauman, 1987) but also has been associated with decreased reproductive performance (Morbeck et al., 1991; Zhao et al., 1992). Such effects on fertility may be caused by lower estrous detection rates in GH-treated cows (Cole et al., 1992; Kirby et al., 1997; Morbeck et al., 1991). In two experiments where lactating dairy cows received their first service by timed artificial insemination, thus eliminating effects of GH on estrous detection, GH administration increased pregnancy rates (Moreira et al., 2000d; Moreira et al., 2001). Magnitude of the increase in pregnancy rates caused by GH was similar whether GH treatment was initiated before or at the time of insemination (Moreira et al., 2001). Similarly, increased pregnancy rates were observed in multiparous cows having more than one service when a single dose of sustained-release GH was administered at insemination (Hernandez-Ceron et al., 2000). Treatment of superovulated donor cows with GH at insemination decreased the number of unfertilized oocytes and increased the percentage of transferable embryos and the percentage of embryos in more

advanced stages of development (see Chapter 4). The ability of GH to increase fertilization rates and enhance early embryonic development may be mediated directly by GH, or indirectly, via IGF-I, whose circulating concentrations are increased after administration of GH (Peel and Bauman, 1987).

Objectives of this experiment were to test whether addition of GH to the maturation medium and GH or IGF-I to the culture medium of a bovine in vitro fertilization system affects oocyte maturation and early embryonic development. The experiment also was designed to examine whether potential effects of GH during embryo culture are independent from IGF-I.

MATERIALS AND METHODS

Materials

Lyophilized recombinant bovine GH was obtained from Monsanto (St. Louis, MO) and reconstituted in a 0.01 M $NaHCO_3$ solution. Lyophilized recombinant human IGF-I (Upstate Biotechnology; Lake Placid, NY) was reconstituted in 0.1 M acetic acid. Lyophilized mouse monoclonal IgG_1 antibody to human IGF-I (Upstate Biotechnology; Lake Placid, NY) was reconstituted in sterile water. A monoclonal mouse IgG_1 antibody to heat stress protein 65 of

Mycobacterium bovis diluted in PBS (StressGen Biotechnologies Corp.; Victoria, Canada) was used as a control antibody. After reconstitution, reagents were aliquoted, and stored at - 70 °C. Essentially fatty-acid free BSA was purchased from Sigma Chemical Company (St. Louis, MO). Bovine steer serum was acquired from Pel Freez (Rogers, AR). Modified Tyrode's solutions (Specialty Media; Lavallette, NJ) were used to prepare HEPES-Tyrode's albumin lactate pyruvate (TALP), in vitro fertilization (IVF)-TALP, and Sperm-TALP (Parrish et al., 1989). FSH was purchased from AgTech (Folltropin; Manhattan, KA) and Percoll from Amersham Pharmacia Biotech (Piscataway, NJ). Frozen semen from various bulls was obtained from Select Sires (Plain City, OH) and American Breeders Service (Madison, WI). Tissue Culture Medium (TCM-199), MEM nonessential amino acids and BME essential amino acids were obtained from Sigma and KSOM was purchased from Specialty Media(Phillipsburg, NJ). Hoechst 33342 dye was acquired from Sigma. Mounting medium containing an antifade solution was purchased from Molecular Probes (Eugene, OR). Other reagents were purchased from Fischer Scientific (Pittsburgh, PA) or Sigma.

In Vitro Production of Embryos

Experimental procedures used during in vitro maturation, fertilization, and culture were similar o methods described previously by Paula-Lopes et al. (1998). Ovaries from slaughtered cows were washed several times with saline (0.9% [w:v] NaCl) containing 100 U/mL penicillin-G and 100 µg/mL streptomycin at 25 to 30 °C to remove blood and debris. Cumulus-oocyte complexes (COCs) were obtained by slicing the ovary. The COCs were washed three times in oocyte collection medium (TCM-199 supplemented with 2% [v:v] steer serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 15 mg/mL glutamine and 0.04 USP U/mL heparin). Groups of approximately 10 oocytes were placed in 50-µL drops of oocyte maturation medium (TCM-199 supplemented with 22 µg/mL pyruvate, 20 µg/mL FSH, 2 µg/mL estradiol, and 50 µg/mL gentamicin) and covered with mineral oil. The COCs were matured for 22 h at 38.5 °C in an atmosphere of 5% CO_2 in humidified air.

Frozen-thawed sperm (n = 3 straws, each from a different bull selected at random) were purified by centrifugation on a Percoll gradient (3 mL of 45% Percoll [v:v] over 3 mL of 90% Percoll) at 2000 x *g* for 20 min. The sperm pellet was collected at the bottom of the tube and placed in 10 mL of Sperm-TALP. Spermatozoa were

centrifuged at 1000 x *g* for 5 min. The supernatant was removed, and the viable sperm pellet was resuspended in IVF-TALP to give an approximate concentration of 25 million spermatozoa/mL.

After maturation, COCs were removed from maturation drops and washed once in HEPES-TALP. Groups of approximately 30 oocytes were transferred to four-well plates containing 600 µL of IVF-TALP per well. Oocytes were fertilized with 25 µL of sperm suspension (approximately 6.2 x 10^5 cells) and 25 µL of PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% [w:v] NaCl) added to each well. After 8 to 10 h at 38.5 °C and 5% CO_2 in humidified air, presumptive zygotes were removed from fertilization wells, placed in microcentrifuge tubes containing 40 µL HEPES-TALP, and vortexed for 5 min. Presumptive zygotes were washed 2 times in HEPES-TALP to remove remaining cumulus cells and associated spermatozoa. Although most of the cumulus cells were removed by vortexing and washing oocytes, in some cases a few cells were present during embryo culture.

Groups of approximately 10 presumptive zygotes were placed in 25 µL drops of KSOM supplemented with 2.0 mg/mL of essentially fatty-acid free BSA, 0.5 µl/mL gentamicin, MEM non-essential amino acid solution, and BME amino acids

solution and maintained at 38.5 °C and 5% CO_2 in humidified air throughout the 8-d period of embryo culture.

Embryo Fixation and Determination of Cell Number

After culture, embryos that developed to the blastocyst stage were removed from the culture medium and placed in 4% paraformaldehyde for 1 h, washed in PBS containing 1 mg/mL polyvinylpyrrolidone, and affixed to poly-1-lysine coated microscope slides. Embryos were covered with 1 μg/mL of Hoechst dye for 5 min at ambient temperature. Excess Hoechst dye 33342 was removed by blotting, a coverslip was mounted using a mounting medium containing an antifade solution (Molecular Probes; Eugne, OR), and fluorescent staining of nuclei visualized with an epifluorescence microscope.

Experimental Treatments

A total of 14 treatments were added to the maturation and culture media in a 2x7 factorial arrengement(Table 5.1). At maturation, either GH or the same volume of 0.01 M $NaHCO_3$ (i.e., the diluent for GH; control) was added to the medium. During embryo culture, one of the seven treatments depicted in Table 5.1 were added to the culture medium at d 0 (i.e., first day of culture). The antibody raised to heat shock protein 65 of *Mycobacterium bovis* was

included as a control for effect of the antibody raised against to IGF-I. The experiment was replicated six times. Oocytes were assigned randomly to maturation medium treatments and inseminated oocytes were assigned randomly to culture medium treatments blocked within maturation treatment.

Table 5.1. Experimental treatments during maturation and embryo culture and number of inseminated oocytes per group.

Culture treatment	Maturation treatment	
	Control	GH[2]
Control	1) Control-control n = 121	8) GH-control n = 130
IgG[1]	2) Control-IgG n = 120	9) GH-IgG n = 129
GH[2] + IgG	3) Control-GH/IgG n = 131	10) GH-GH/IgG n = 132
IGF-I[3] + IgG	4) Control-IGF/IgG n = 130	11) GH-IGF/IgG n = 121
Anti-IGF-I[4]	5) Control-anti-IGF n = 110	12) GH-anti-IGF n = 141
GH + Anti-IGF-I	6) Control-GH/anti-IGF n = 132	13) GH-GH/anti-IGF n = 122
IGF-I + Anti-IGF-I	7) Control-IGF/anti-IGF n = 129	14) GH-IGF/anti-IGF n = 130

[1] Final concentration was 10 µg/mL.
[2] Final concentration was 100 ng/mL.
[3] Final concentration was 100 ng/mL.
[4] Final concentration was 10 µg/mL.

Cleavage rates were assessed at d 3 postinsemination and calculated as the percentage of inseminated oocytes that developed to the 2-cell stage or greater. Development to the blastocyst stage was determined at d 7 and at d 8 after insemination. At these times, blastocysts were classified as non-expanded (i.e., early and normal blastocysts) or in advanced stages of development (i.e., expanded and hatched blastocysts). Results were expressed as a percentage of inseminated oocytes that became blastocysts and also as a percentage of cleaved oocytes that developed to the blastocyst stage at both d 7 and d 8 of embryo culture.

Statistical Analyses

Experimental responses were analyzed using the method of least squares analysis of variance (ANOVA) of the General Linear Model (GLM) procedure of the Statistical Analysis System software package (SAS, 1988). Each drop was considered as an experimental unit. Three different methods were utilized for data analyses. In Method 1, percentages of cleaved embryos or embryos that developed to the blastocyst stage were considered as the dependent variable and the mathematical model included effects of treatment, replicate, and the interaction between treatment

and replicate. In Method 2, percentage data were analyzed after arcsin transformation with a similar mathematical model. In Method 3, the number of cleaved embryos and the number of blastocysts per drop were considered to be the dependent variables and the total number of presumptive embryos in a drop was used as a covariate. This method avoids the nonestimable bias for percentage data due to different number of observations per drop. Similar results were obtained from the three methods and results were expressed as the percentage of cleaved embryos or embryos that developed to the blastocyst stage. The effect of treatments on embryo cell number also was analyzed by ANOVA of the General Linear Model (GLM) procedure of SAS (SAS, 1988) and each embryo was considered as the experimental unit.

Differences among treatment groups were determined by thirteen pre-established orthogonal contrasts. Contrasts (n = 7) that examined the main effects of treatments are depicted in Table 5.2. The remaining contrasts (n = 6) examined the interactions between maturation and culture treatments. Differences were considered significant at $P < 0.05$.

Table 5.2. Description of the orthogonal contrasts that examined main effects of treatments.

Contrasts	Treatment comparisons
Maturation treatments	
1) GH versus control	Treatments 1 to 7 versus treatments 8 to 14
Culture treatments	
2) Control versus IgG and anti-IGF	Treatments 1, 8 versus treatments 2, 5, 9, 12
3) IgG versus Anti-IGF	Treatments 2, 9 versus treatments 5, 12
4) IgG versus GH/IgG and IGF/IgG	Treatments 2, 9 versus treatments 3, 4, 10, 11
5) GH/IgG versus IGF/IgG	Treatments 3, 10 versus treatments 4, 11
6) Anti-IGF versus GH/anti-IGF and IGF/anti-IGF	Treatments 5, 12 versus treatments 6, 7, 13, 14
7) GH/anti-IGF versus IGF/anti-IGF	Treatments 6, 13 versus treatments 7, 14

RESULTS

Least squares means for cleavage rates at d 3 and development to blastocyst at d 7 and d 8 of embryo culture among treatment groups are depicted in Table 5.3. Overall cleavage rate and development to blastocyst at d 7 and d 8 for the experiment were 85.6% (1522/1778), 12.5% (222/1778), and 22.1% (393/1778), respectively.

Table 5.3. Cleavage rates, development to blastocyst at d 7 and at d 8 of culture as a percentage of total inseminated oocytes for treatment groups (LSM ± SE).

Treatment groups	Cleavage rates (%)	Blastocyst at d 7 (%)	Blastocyst at d 8 (%)
Control-control	78.5 ± 3.5	9.2 ± 2.8	16.6 ± 3.6
Control-IgG	87.5 ± 3.5	10.9 ± 2.8	19.1 ± 3.6
Control-GH/IgG	83.9 ± 3.4	17.1 ± 2.7	24.9 ± 3.5
Control-IGF/IgG	86.9 ± 3.2	15.8 ± 2.5	29.7 ± 3.3
Control-anti-IGF	85.0 ± 3.5	10.8 ± 2.8	19.9 ± 3.6
Control-GH/anti-IGF	79.9 ± 3.4	12.7 ± 2.7	22.3 ± 3.5
Control-IGF/anti-IGF	84.0 ± 3.4	12.4 ± 2.7	18.9 ± 3.5
GH-control	89.4 ± 3.4	8.5 ± 2.7	18.3 ± 3.5
GH-IgG	82.8 ± 3.4	5.8 ± 2.7	12.8 ± 3.5
GH-GH/IgG	87.8 ± 3.4	15.1 ± 2.7	24.7 ± 3.5
GH-IGF/IgG	91.0 ± 3.5	16.6 ± 2.8	36.5 ± 3.6
GH-anti-IGF	88.2 ± 3.3	11.6 ± 2.6	20.8 ± 3.4
GH-GH/anti-IGF	84.6 ± 3.5	16.5 ± 2.8	25.7 ± 3.6
GH-IGF/anti-IGF	86.9 ± 3.5	8.7 ± 2.8	15.3 ± 3.6

Orthogonal contrasts indicated that cleavage rates were affected by GH treatment during maturation. Oocytes matured with GH had greater ($P < 0.05$) cleavage rates than control groups (87.3 ± 1.2% > 83.9 ± 1.2%). In addition, an interaction between treatments at maturation and control versus IgG and anti-IGF treatments at culture was detected ($P < 0.05$). In the absence of GH during maturation, cleavage rates of control/IgG (87.5 ± 3.5%) and control/anti-IGF (85.0 ± 3.5%) were greater than cleavage rates for control-control (78.5 ± 3.5%). For oocytes matured with GH, no differences among control and antibody groups were observed (GH-control = 89.4 ± 3.4%; GH-IgG = 82.8 ± 3.4%; GH-anti-IGF = 88.2 ± 3.3%). There were no other significant contrasts for cleavage rates. Cleavage rates varied among replicates from 80.9 ± 2.1% to 91.2 ± 2.3% ($P < 0.04$), but there was no interaction between treatments and replicate.

Blastocyst development at d 7 of embryo culture as a percentage of total inseminated oocytes was not affected by maturation treatments (control = 12.7 ± 1.0% and GH = 12.1 ± 1.0%) or by an interaction between oocyte maturation and embryo culture treatments. However, blastocyst development of groups treated with GH/IgG (16.2 ± 1.8%) and IGF/IgG

(16.5 ± 1.8%) was increased ($P < 0.01$) compared to IgG groups (8.7 ± 1.4%). Remaining contrasts were not significant. An effect of replicate was detected ($P < 0.01$) and blastocyst development at d 7 varied from 8.0 ± 1.8% to 18.7 ± 1.7%. An interaction between treatments and replicate was not observed.

At d 8 after insemination, blastocyst development as a percentage of inseminated oocytes was not influenced by treatments during maturation (control = 21.6 ± 1.4% and GH = 22.6 ± 1.4%) and there was no interaction between treatments during oocyte maturation and embryo culture. Thus, data was pooled according to treatments during embryo culture. Development to the blastocyst stage at d 8 was affected by treatment during embryo culture. Blastocyst development was greater ($P < 0.01$) for GH/IgG and IGF/IgG groups compared to IgG groups (Figure 5.1) and was greater ($P < 0.02$) for IGF/IgG than for GH/IgG (Figure 5.1). Blastocyst development did not differ between anti-IGF groups compared to GH/anti-IGF and IGF/anti-IGF groups, but development was greater ($P < 0.05$) for GH/anti-IGF than for IGF/anti-IGF (Figure 5.1). All other contrasts were not significant. Development to the blastocyst stage at d 8 varied among replicates from 15.4 ± 2.3% and 28.1 ± 2.2% (P

< 0.01), but there were no interactions between treatment and replicate.

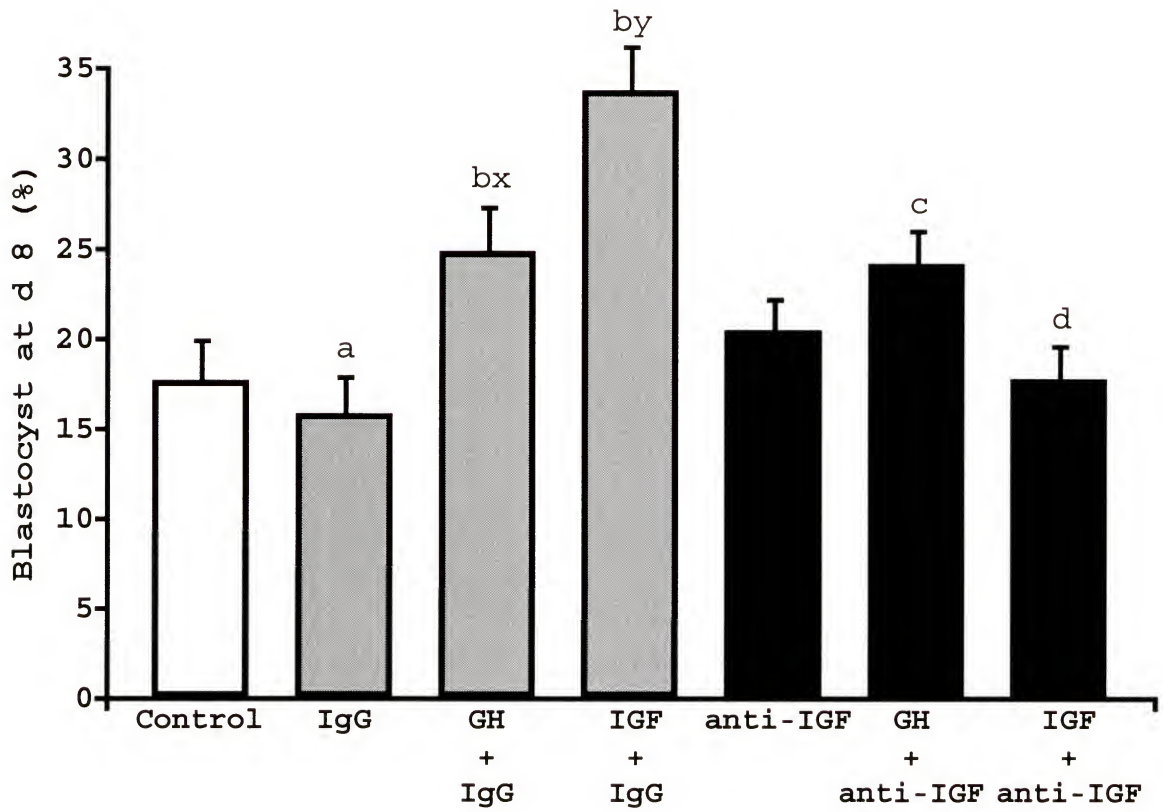

Figure 5.1. Percentage of inseminated oocytes that developed to blastocysts at d 8 of embryo culture (LSM ± SE). Controls are represented by an open box, IgG treatments by light gray boxes, and anti-IGF-I groups by black boxes. Different letters indicate significant orthogonal contrasts: a versus b, P < 0.01; x versus y, P < 0.02; c versus d, P < 0.05.

Development to the blastocyst stage at d 7 and 8 of culture was reexamined in subsequent analyses as a percentage of cleaved embryos (Table 5.4). Overall development to blastocyst at d 7 and at d 8 of culture as a

percentage of cleaved oocytes were 14.6% (222/1522) and 25.8% (393/1522), respectively.

Table 5.4. Development to blastocyst at d 7 and at d 8 of embryo culture as a percentage of cleaved embryos (LSM ± SE).

Treatment groups	Blastocyst at d 7 (%)	Blastocyst at d 8 (%)
Control-control	11.6 ± 3.2	20.6 ± 4.1
Control-IgG	12.2 ± 3.2	21.3 ± 4.1
Control-GH/IgG	21.1 ± 3.1	31.3 ± 4.0
Control-IGF/IgG	18.2 ± 2.9	33.9 ± 3.8
Control-anti-IGF	13.2 ± 3.3	24.1 ± 4.2
Control-GH/anti-IGF	16.5 ± 3.1	29.5 ± 4.0
Control-IGF/anti-IGF	14.3 ± 3.1	22.3 ± 4.0
GH-control	9.4 ± 3.1	20.6 ± 4.0
GH-IgG	6.8 ± 3.1	15.8 ± 4.0
GH-GH/IgG	17.1 ± 3.1	27.3 ± 4.0
GH-IGF/IgG	18.4 ± 3.2	40.5 ± 4.1
GH-anti-IGF	13.6 ± 3.0	23.5 ± 3.9
GH-GH/anti-IGF	19.9 ± 3.2	31.0 ± 4.1
GH-IGF/anti-IGF	10.9 ± 3.3	19.3 ± 4.2

Orthogonal contrasts indicated that treatments at maturation did not affect development to the blastocyst stage at d 7 of culture (control = 15.1 ± 1.2% and GH = 14.0 ± 1.2%). Also, there was no interaction between treatments during oocyte maturation and embryo culture.

Treatments at initiation of embryo culture influenced development to blastocyst at d 7. Groups treated with GH/IgG and IGF/IgG had greater development to blastocyst than IgG groups (18.9 ± 2.1% and 18.6 ± 2.1% > 9.6 ± 2.2%). Although there were no differences in blastocyst development between anti-IGF (13.6 ± 2.1%), GH/anti-IGF (18.2 ± 2.2%), and IGF/anti-IGF (13.4 ± 2.2%), a trend ($P < 0.09$) suggested that development was increased for GH/anti-IGF compared to IGF/anti-IGF. No other contrasts were significant. Development to the blastocyst stage varied from 8.8 ± 2.1% to 22.0 ± 1.9% according to replicate ($P < 0.01$), but there was no interaction between treatment and replicate.

At d 8 after insemination, treatments during maturation did not affect the percentage of cleaved embryos that developed to blastocyst (control = 25.9 ± 1.6% and GH = 26.0 ± 1.6%) and no interactions between maturation and culture treatments were detected. Development to blastocyst was greater for GH/IgG and IGF/IgG than for IgG groups ($P < 0.01$) and greater for IGF/IgG than for GH/IgG groups ($P < 0.05$; Figure 5.2). Moreover, blastocyst development did not differ among anti-IGF, GH/anti-IGF, and IGF/anti-IGF groups, but it was greater ($P < 0.02$) for GH/anti-IGF than for IGF/anti-IGF groups (Figure 5.2). No

other contrasts were significant. Blastocyst development varied among replicates from 17.0 ± 2.7% to 33.7 ± 2.5% ($P < 0.01$). A treatment by replicate interaction was not detected.

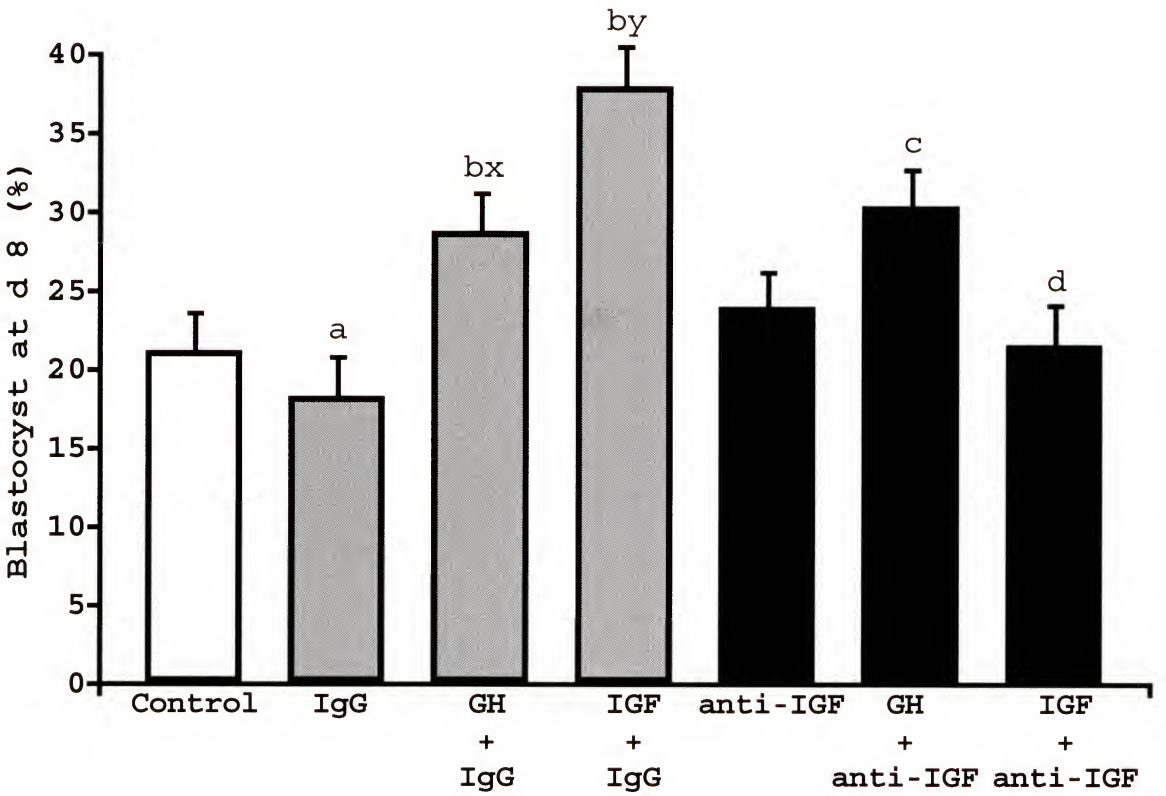

Figure 5.2. Percentage of cleaved embryos that developed to blastocysts at d 8 of embryo culture (LSM ± SE). Controls are represented by an open box, IgG treatments by light gray boxes, and anti-IGF-I groups by black boxes. Different letters indicate significant orthogonal contrasts: a versus b, $P < 0.01$; x versus y, $P < 0.05$; c versus d, $P < 0.02$.

Table 5.5 depicts the percentage of blastocysts that were expanded or hatched (i.e., in advanced stages of

development) at d 7 and 8 of embryo culture and cell number of d-8 embryos that develop to the blastocyst stage.

Table 5.5. Percentage of blastocysts in advanced stages of development (i.e., expanded and hatched blastocysts) at d 7 and 8 of embryo culture and number of cells of d-8 blastocysts (LSM ± SE).

Treatment groups	Advanced at d 7 (%)	Advanced at d 8 (%)	Number of cells
Control-control	15.6 ± 12.6	36.5 ± 11.0	98.5 ± 10.7
Control-IgG	8.9 ± 11.0	42.2 ± 10.9	100.5 ± 10.0
Control-GH/IgG	32.3 ± 8.9	68.8 ± 10.4	126.5 ± 9.5
Control-IGF/IgG	29.0 ± 8.5	52.0 ± 9.6	119.1 ± 7.8
Control-anti-IGF	13.3 ± 10.3	26.5 ± 10.4	105.8 ± 7.7
Control-GH/anti-IGF	36.4 ± 9.3	42.1 ± 10.0	125.7 ± 9.8
Control-IGF/anti-IGF	15.8 ± 9.7	28.8 ± 10.0	104.3 ± 10.1
GH-control	3.7 ± 11.0	19.1 ± 10.5	87.0 ± 8.9
GH-IgG	1.0 ± 11.0	26.4 ± 11.0	93.5 ± 10.7
GH-GH/IgG	23.5 ± 9.3	49.5 ± 9.6	120.8 ± 9.7
GH-IGF/IgG	12.1 ± 9.9	51.2 ± 12.4	141.0 ± 10.4
GH-anti-IGF	14.5 ± 9.9	37.8 ± 10.5	100.6 ± 7.2
GH-GH/anti-IGF	32.6 ± 10.3	50.1 ± 10.4	128.1 ± 9.4
GH-IGF/anti-IGF	16.6 ± 10.4	29.3 ± 10.1	109.7 ± 9.7

Orthogonal contrasts did not detect an effect of treatment at maturation (control = 23.2 ± 3.8% and GH = 15.7 ± 4.0%) or of an interaction between maturation and culture treatments on the percentage of blastocysts in advanced stage of development at d 7. Addition of GH/IgG

and of IGF/IgG during embryo culture increased ($P < 0.03$) the percentage of blastocysts in advanced stages of development compared to IgG (28.3 ± 5.7% and 23.0 ± 5.6% > 5.4 ± 7.2%). Percentage of blastocysts in advanced stage of development was similar for anti-IGF (10.3 ± 8.1%) compared to GH/anti-IGF (35.6 ± 7.8%) and IGF/anti-IGF groups (15.6 ± 7.9%). A trend ($P < 0.06$) suggested that the percentage of blastocysts in advanced stage of development was greater for GH/anti-IGF than for IGF/anti-IGF.

No effect of treatments during maturation (control = 42.5 ± 4.0% and GH = 37.5 ± 4.2%) or an interaction between oocyte maturation and embryo culture treatments were detected for the percentage of blastocysts in advanced stage of development at d 8. A greater ($P < 0.02$) percentage of blastocysts were in advanced stage of development for GH/IgG and IGF/IgG than for IgG groups (Figure 5.3). There was also a trend ($P < 0.09$) for greater percentage of blastocysts in advanced stage of development in GH/anti-IGF compared to IGF/anti-IGF groups. However, there were no differences between the GH/anti-IGF and IGF-anti-IGF groups compared to anti-IGF groups (Figure 5.3).

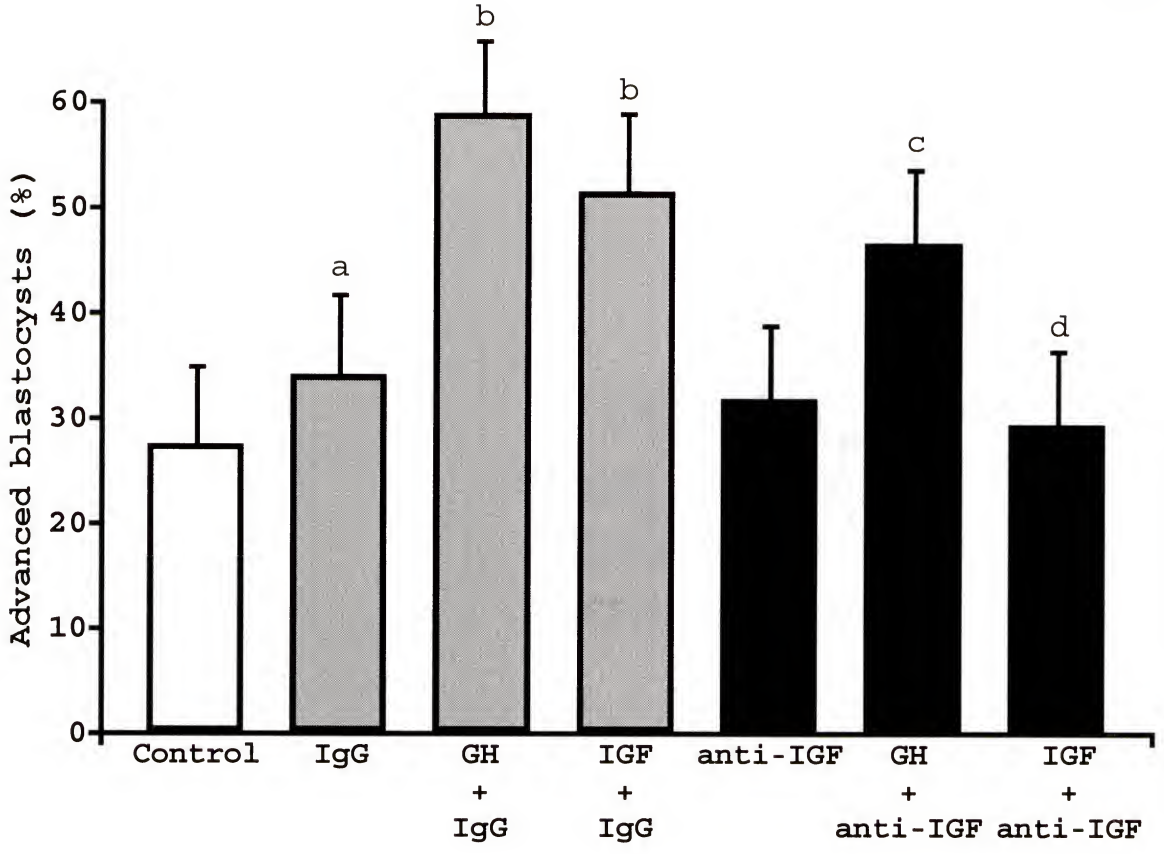

Figure 5.3. Percentage of d-8 blastocysts in advanced stages of development (i.e., expanded and hatched blastocysts; LSM ± SE). Controls are represented by an open box, IgG treatments by light gray boxes, and anti-IGF-I groups by black boxes. Different letters indicate significant orthogonal contrasts: a versus b, $P < 0.02$; c versus d, $P < 0.09$.

Cell number of blastocysts at d 8 after insemination was not influenced by treatments during maturation (control = 113.2 ± 3.5 and GH = 109.7 ± 3.5) or by an interaction between treatments during oocyte maturation and embryo culture. Number of cells of d-8 blastocysts was increased ($P < 0.1$) for GH/IgG and IGF/IgG groups compared to IgG groups (Figure 5.4). In addition, number of cells of d-8

blastocysts tended to be greater ($P < 0.06$) for GH/anti-IGF and IGF/anti-IGF groups than for anti-IGF groups, and was greater ($P < 0.04$) for GH/anti-IGF groups than for IGF/anti-IGF groups (Figure 5.4).

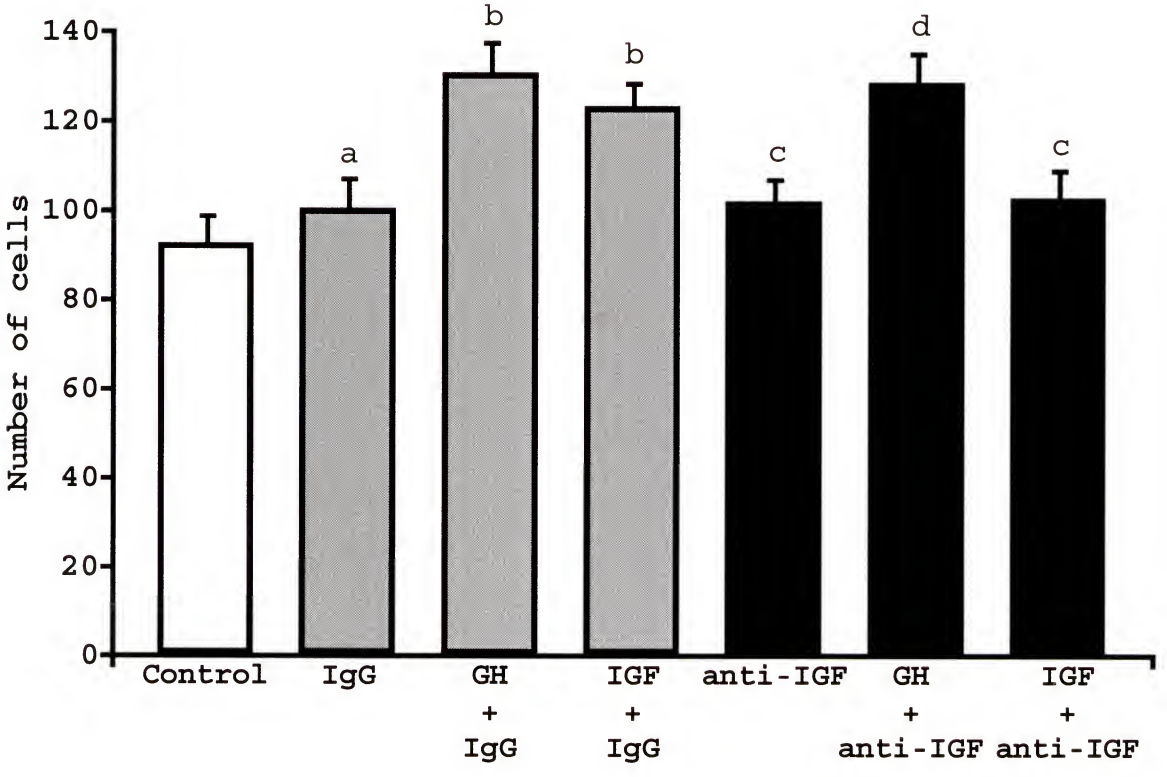

Figure 5.4. Number of cells of d-8 blastocysts (LSM ± SE). Controls are represented by an open box, IgG treatments by light gray boxes, and anti-IGF-I groups by black boxes. Different letters indicate significant orthogonal contrasts: a versus b, $P < 0.01$; c versus d, $P < 0.04$.

DISCUSSION

Results indicated that addition of GH to the maturation medium increased cleavage rates of bovine oocytes in vitro and that development to the blastocyst stage, percentage of blastocysts in advanced stages of

development and number of cells per blastocyst were increased by GH and IGF-I treatments. Addition of an antibody to IGF-I to embryo culture medium inhibited the effects of IGF-I but did not eliminate the effects of GH on early embryonic development. Thus, GH affects embryonic development independently from IGF-I.

Addition of GH to the maturation treatment increased the percentage of oocytes undergoing cleavage at d 3 after insemination. Previous reports indicated that GH stimulates maturation of bovine oocytes and subsequent fertilization and cleavage rates (Izadyar et al., 1996; Izadyar et al., 1998a; Izadyar et al., 1998b). In cattle, both GH mRNA and GH protein are produced by granulosa, theca, and cumulus cells within ovarian follicles and by the oocyte (Izadyar et al., 1999), and GH receptors were identified in both cumulus cells and oocyte (Izadyar et al., 2000; Kolle et al., 1998). This suggests a possible paracrine and/or autocrine role for GH during oocyte maturation. An initial experiment suggested that GH effects on oocyte maturation were expressed through cumulus cells (Izadyar et al., 1997b). Subsequently, it was demonstrated that GH also stimulates maturation of denuded oocytes (Izadyar et al., 1997a; Izadyar et al., 1998b). Furthermore, GH can affect oocyte maturation independently

from IGF-I and through the cAMP signal transduction pathway (Izadyar et al., 1997a; Izadyar et al., 1997b).

Nonetheless, the magnitude of the increase in cleavage rates due to GH treatment was numerically lower in the present experiment (approximately 3.4%) than in previous reports where cleavage rates increased between 12.5 to 19.0% (Izadyar et al., 1996; Izadyar et al., 1998b). Such a discrepancy may be caused by differences in the in vitro maturation systems. Cleavage rates in the present experiment averaged 85.6% whereas cleavage rates ranged from 56 to 59% for controls and from 68.5 to 78% in GH treated groups in previous reports (Izadyar et al., 1996; Izadyar et al., 1998b). Because cleavage rates for control-treated inseminated oocytes in the present experiment are already high, further increases in cleavage rates were likely to be of lower magnitude. In addition, FSH also was added to maturation medium in the present experiment. In another experiment, presence of FSH in the maturation medium increased cleavage rates and eliminated the stimulatory effects of GH (Izadyar et al., 1998b).

In contrast with previous reports (Izadyar et al., 1996; Izadyar et al., 1998b), addition of GH during oocyte maturation did not affect development to the blastocyst stage. Also, no interactions between treatments during

oocyte maturation and embryo culture on blastocyst development were observed. This supports the observation that addition of GH at maturation did not affect the developmental capacity of embryos after fertilization. The increase in development to blastocyst observed in the two previous experiments probably was caused by increased cleavage rates and not by a subsequent effect on the developmental capacity of fertilized oocytes. This is so because development to blastocyst as a percentage of cleaved oocytes were similar for control and GH treatments: 34.2% (144/420) for GH as compared to 30.2% (102/338) for controls, (calculated from Izadyar et al., 1996), and 38.1% (107/281) for GH-treated oocytes versus 34.5% (80/232) for control-treated oocytes (calculated from Izadyar et al., 1998b).

Cleavage rate was affected by an interaction between treatments during oocyte maturation and embryo culture. Among control groups at maturation, addition of antibodies (i.e., IgG and anti-IGF-I) to embryo culture medium increased subsequent cleavage rates. This was an unexpected result and the reasons for such an effect are not clear. A direct effect of the antibodies to stimulate cleavage is not likely, especially in the case of the control IgG, which was raised to a bacterial protein and

not expected to interact with bovine embryos. Perhaps, the increase in protein concentration of the maturation medium by addition of immunoglobulins induced a small but significant increase in cleavage rates. Nonetheless, the effect of adding immunoglobulins at culture on cleavage rates was not observed when oocytes were matured in the presence of GH. Addition of GH at maturation may have stimulated oocytes to approximate their maximum fertilization capacity, which could not be further increased by higher protein concentrations in the medium. It is also important to note that neither GH nor IGF-I affected cleavage rates when added during embryo culture and, therefore, did not affect the capacity of fertilized oocytes to undergo cleavage.

Addition of GH or IGF-I to the culture medium stimulated blastocyst development at d 7 and at d 8 of embryo culture. Previous reports also have suggested that addition of GH or IGF-I to embryo culture stimulates embryonic development to the blastocyst stage (Izadyar et al., 2000; Kaye et al., 1992; Palma et al., 1997). The percentage of blastocysts that were classified in advanced stages of development (i.e., expanded and hatched blastocysts) increased due to GH and IGF-I treatments at d 7 and 8 after insemination. In addition, the number of

cells of d-8 blastocysts was increased in GH and IGF-I treatments compared to IgG controls. In another study, addition of both IGF-I and IGF-II increased the number of cells and decreased the number of apoptotic cells during in vitro culture of bovine embryos (Byrne et al., 1999). Therefore, addition of GH or IGF-I to culture medium not only increased the number of embryos to reach the blastocyst stage, but also accelerated the rate of development of these embryos.

Effects of GH and IGF-I on embryonic development are probably mediated directly because both GH and IGF-I receptors are expressed by the early embryo (Izadyar et al., 2000; Watson et al., 1992). Furthermore, concentrations of IGF-I in the oviductal lumen are increased during the initial stages of development (Schmidt et al., 1994) and both GH and IGF-I are produced by the early embryo (Lechniak et al., 1999; Lonergan et al., 2000). Fertilized oocytes that cleaved < 30 h after insemination expressed mRNA for IGF-I and had a greater development to blastocyst whereas fertilized oocytes that cleaved > 36 h after insemination did not express IGF-I mRNA and had a lower development to the blastocyst stage (Lonergan et al., 1999; Lonergan et al., 2000). Collectively, these observations suggest that there may be

a paracrine or autocrine mechanism through which GH and IGF-I stimulate early embryonic development.

Interpretation of experimental results indicates that GH stimulated embryonic development independently from IGF-I. Addition of anti-IGF-I during embryo culture eliminated the effects of IGF-I on development to blastocyst stage. Similarly, the percentage of blastocysts classified as expanded or hatched and the number of d-8 blastocysts were not affected by IGF-I when embryos were cultured in the presence of anti-IGF-I. However, the inhibitory effect of anti-IGF-I was not exerted when embryos were co-cultured in the presence of GH. An independent role for GH was previously observed on oocyte maturation (Izadyar et al., 1997b). In mice, GH enhances embryonic development during culture, but this effect was neutralized by a GH receptor antibody (Fukaya et al., 1998). When the GH receptor gene was knocked out, reproductive performance of both male and female mice was reduced (Chandrashekar et al., 1999; Danilovich et al., 1999). Therefore, GH may exert a direct and more significant effect on the reproductive system than previously assumed.

Present observations support results from Chapters 3 and 4 and other in vivo experiments (Hernandez-Ceron et al., 2000; Moreira et al., 2000d) where administration of

GH improved fertilization rates, early embryonic development, and pregnancy rates following artificial insemination or embryo transfer. The findings here and elsewhere that GH improves oocyte maturation and their capacity to undergo fertilization are consistent with observations that administration of GH to superovulated cows at insemination decreased the number of unfertilized ova and increased the percentage of transferable embryos (Chapter 4). In addition, increased fertilization rates may be in part responsible for the increased pregnancy rates of lactating dairy cows treated with exogenous GH as reported in Chapter 3 and in the literature (Hernandez-Ceron et al., 2000; Moreira et al., 2000d). Similarly, the observation that GH and IGF-I advanced the stage of development and the number of cells of bovine embryos in vitro may explain the increased pregnancy rates of recipient cows transferred with frozen-thawed embryos which early development was influenced by exogenous GH (Chapter 4). It is possible that GH treatment may have increased the number of cells of embryos flushed after superovulation and, as a consequence, increased their capacity to survive the freezing and thawing process. Also, the stimulatory effects of GH and IGF-I on early embryonic development may explain in part the increased pregnancy rates of lactating

dairy cows treated with GH at around the time of ovulation (Chapter 3; Hernandez-Ceron et al., 2000; Moreira et al., 2000d).

CONCLUSIONS

In conclusion, addition of GH to the maturation medium increased cleavage rates of bovine oocytes in vitro. Development to the blastocyst stage, rate of embryonic development and number of cells per blastocyst were increased by GH and IGF-I treatments. An antibody to IGF-I inhibited the effects of IGF-I when added to culture medium, but did not eliminate the effects of GH on early embryonic development. These findings support observations made in Chapters 3 and 4.

There is an increasing amount of evidence that indicate that GH may enhance fertility of cattle either directly or through the IGF-I system. Further research is warranted to investigate the mechanisms by which administration of exogenous GH may increase fertility of cows following artificial insemination or embryo transfer.

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSIONS

Results from the experiment described in Chapter 3 indicated that pre-synchronization increased first-service pregnancy rates of lactating dairy cows submitted to the Ovsynch/TAI protocol. The experimental design does not allow for any conclusive indication of whether such an effect of pre-synchronization was due to an effect of $PGF_{2\alpha}$ on uterine health or number of estrous cycles prior to insemination. Nonetheless, data obtained allows for the conclusion that pre-synchronization altered the distribution of cows according to stage of the estrous cycle at initiation of the Ovsynch/TAI protocol based on P_4-classes. Furthermore, data also indicated that such a change in the distribution of cows according to stage of the cycle had a positive effect on subsequent pregnancy rates. This constitutes further evidence to support the hypothesis that stage of the estrous cycle at initiation of the Ovsynch/TAI protocol does affect pregnancy rates (Moreira et al., 2000b). The observation that pre-synchronizing cows prior to initiation of the Ovsynch/TAI

protocol to target the first GnRH injection to the early diestrous phase of the estrous cycle increases pregnancy rates raises further questions. One clear effect of pre-synchronization is that it decreased the percentage of cows that had premature CL regression. Cows that regress their CL prior to the $PGF_{2\alpha}$ injection of the Ovsynch/TAI protocol are at a greater risk to ovulate before the time of insemination (Moreira et al., 2000b). It can be expected that such an asynchrony of ovulation leads to decreased chances of conception to the timed insemination service and reduces pregnancy rates, as documented previously (Moreira et al., 2000d; Stevenson et al., 1999; Vasconcelos et al., 1999b).

Another possible explanation for the observed increased pregnancy rates is that ovulation and follicular synchronization induced by the first GnRH injection of the Ovsynch/TAI protocol was increased after pre-synchronization. Although such an effect was not as evident compared to the decrease in premature CL regression, data presented in Chapter 3 further support this hypothesis. Plasma P_4 concentrations at $PGF_{2\alpha}$ (i.e., 7 d after the first GnRH injection) of pre-synchronized cows were increased compared to cows not pre-synchronized. It was suggested that cows that underwent pre-synchronization

should have a greater ovulation rate after the first injection of GnRH of the Ovsynch/TAI protocol, which explains the increase in plasma P_4 concentrations. Increased plasma P_4 concentrations at $PGF_{2\alpha}$ were positively correlated with increased pregnancy rates, which indicate that cows that ovulate and form an accessory CL after the first GnRH injection have greater pregnancy rates. In addition, cows determined to have initiated the Ovsynch/TAI protocol at metestrus based on plasma P_4 concentration had lower pregnancy rates compared to cows estimated to have initiated the synchronization program during diestrus. The hypothesis that cows ovulating to the first GnRH injection of the Ovsynch/TAI protocol have greater pregnancy rates also is supported by data in the literature as discussed in Chapter 2.

It is possible that implementation of pre-synchronization systems, other than two injections of $PGF_{2\alpha}$ given 14 days apart, that elicit a tighter synchronization of estrus may further enhance pregnancy rates to the Ovsynch/TAI protocol. One of the reasons for choosing to synchronize cows with two injections of $PGF_{2\alpha}$ given 14 days apart in the experiment described in Chapter 3 was the relatively inexpensive drug cost. Nonetheless, it was expected that cows would be anywhere from d 5 to d 12 of

the cycle at the beginning of the Ovsynch/TAI program. Because emergence of the second-wave follicle occurs approximately at d 9 for two-wave cycles or d 11 for three-wave cycles (Ginther et al., 1989), initiation of the Ovsynch/TAI between days 5 to 8 of the cycle would result in greater ovulation rates than between days 9 to 12 of the cycle. Thus, targeting the initiation of the Ovsynch/TAI protocol to d 5 to 8 instead of d 5 to 12 of the cycle would further increase pregnancy rates.

Pre-synchronization of cows with an injection of GnRH followed 7 d later by $PGF_{2\alpha}$ may induce greater pregnancy rates than pre-synchronization with two injections of $PGF_{2\alpha}$ given 14 days apart. Estrous synchronization after the GnRH-$PGF_{2\alpha}$ injections increased estrous detection rates and elicited a tighter synchronization than an injection of $PGF_{2\alpha}$ (Thatcher et al., 1989; Macmillan and Thatcher, 1991). Even though the use of GnRH-$PGF_{2\alpha}$ is a more expensive method of pre-synchronization, a small increase in pregnancy rates may justify such an investment. Furthermore, other advantages associated with this system may be obtained when the incidence of anestrous cows at initiation of the Ovsynch/TAI protocol is taken into consideration.

Results form Chapter 3 clearly demonstrated that pre-synchronization with two injections of $PGF_{2\alpha}$ given 14 days

apart had no effects on the incidence of anestrous cows at initiation of the Ovsynch/TAI protocol and also on subsequent pregnancy rates of anestrous cows. Moreover, pregnancy rates of anestrous cows were reduced when compared to pregnancy rates of cyclic cows. Therefore, a pre-synchronization system that stimulates cyclicity among anestrous cows has the potential to increase pregnancy rates after the Ovsynch/TAI protocol. Data from Chapter 3 indicated that a single injection of GnRH induced an ovulation in 50% of anestrous cows and 75% of anestrous cows ovulated at least once to the two GnRH injections given 9 d apart. It should be expected that such a result would vary depending upon stage of lactation and nutritional status of cows, but pre-synchronization of cows with GnRH-$PGF_{2\alpha}$ would further increase the percentage of cows cycling at initiation of the Ovsynch/TAI protocol.

Other pre-synchronization protocols, based on the use of progesterone or progestins, also may increase pregnancy rates to the Ovsynch/TAI protocol by stimulating anestrous cows to cycle. For instance, cows can be supplemented with exogenous progesterone (e.g., CIDR, MGA) for 7 d and injected with $PGF_{2\alpha}$ at the end of progesterone treatment. Such a protocol has been shown to stimulate cyclicity among anestrous cows (see Chapter 2). Furthermore, in

conjunction with a progesterone treatment, estradiol can be used at initiation and on the day after $PGF_{2\alpha}$ injection to increase responsiveness of anestrous cows and also to enhance the synchrony of estrus/ovulation. One advantage for the inclusion of estradiol is its low cost. The disadvantage of such systems is that neither progesterone nor estradiol (with the exception of estradiol cypionate) is approved to be used lactating dairy cows.

An unexpected observation from the experiment described in Chapter 3 was the high pregnancy rates obtained by LOW-HIGH cows. In Chapter 3, it was discussed that LOW-HIGH cows probably consist of cows in the proestrous or in the early metestrous stage of the estrous cycle. Although cows in early metestrus do not respond to an injection of GnRH, follicular emergence is "naturally" synchronized if cows happen to initiate the Ovsynch/TAI protocol in this particular stage of the cycle. Therefore, subsequent pregnancy rates may not be decreased under such a situation.

However, it was hypothesized that cows that initiate the Ovsynch/TAI protocol during proestrus would be at a greater risk of having incomplete CL regression and, therefore, pregnancy rates would be reduced (Moreira et al., 2000b). In a previous study (Moreira et al., 2000d),

pregnancy rates of LOW-HIGH cows were reduced compared to HIGH-HIGH cows. Differences between experiments may be due to the fact that no distinctions were made between cows that initiated the Ovsynch/TAI during proestrus or early metestrus and anestrous cows. Such a confounding factor was not present in the present experiment because anestrous cows were identified and removed from pregnancy analyses. Results from Chapter 3 indicated that cows that initiate the Ovsynch/TAI protocol during proestrus or early diestrus (i.e., LOW-HIGH cows) did not have an increased incidence of incomplete CL regression after the injection of $PGF_{2\alpha}$ and pregnancy rates were similar to cows initiating the Ovsynch/TAI program during the early diestrus (i.e., HIGH-HIGH cows).

If that is true, it raises the possibility that different schemes of pre-synchronization can be adopted. Long-term supplementation of progesterone (e.g., MGA feeding for 14 to 19 d) can be used to induce persistent dominant follicles after regression of the endogenous CL. An alternative system would be based on the insertion of a CIDR device at d -9, injection of $PGF_{2\alpha}$ at d -2, and, at d 0, CIDR would be removed and the Ovsynch/TAI protocol would be initiated with a GnRH injection. In this manner, the first GnRH injection of the Ovsynch/TAI protocol could be

administered at the last day or the day after the end of progesterone supplementation. Most probably, the injection of GnRH would induce ovulation of such a persistent follicle and synchronize follicular development. Because it appears that the incidence of incomplete CL regression is not increased under this situation, pregnancy rates may be further increased due to a greater synchrony of follicular synchronization.

In conclusion, there are several other methods that can be used to pre-synchronize cows prior to the initiation of the Ovsynch/TAI protocol. A series of alternative methods of pre-synchronization are depicted in Figure 6.1. Further research is necessary to determine the advantages and advantages of the described and other different pre-synchronization programs before specific recommendations can be made to cattle producers. Most probably, there will be no single method that would produce optimal results under different management scenarios. A thorough understanding of the physiological mechanisms involved and of management needs for each individual herd will be paramount during the decision process.

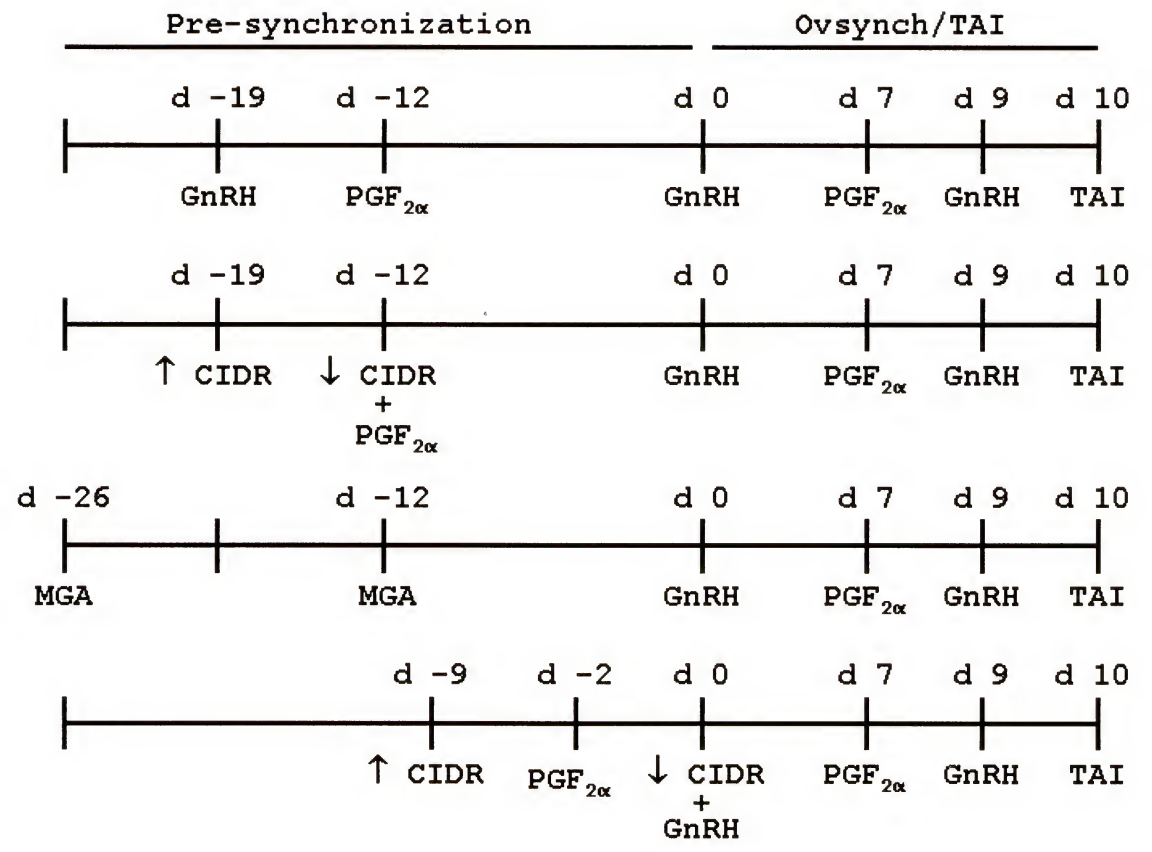

Figure 6.1. Schematic representation of four alternative pre-synchronization strategies. Arrows indicate when CIDR devices would be inserted (↑) or removed (↓).

It was observed in Chapter 3 that pregnancy rates to the second synchronized service were lower when compared to first-service pregnancy rates. Such an observation may be due to the fact that cows received their second service at the beginning of summer and were subjected to heat stress. Nonetheless, an alternative explanation for the decreased pregnancy rates may be related to the stage of the estrous cycle at initiation of the second-service Ovsynch/TAI. In

Chapter 3, cows that did not conceive to the first-service were re-synchronized 32 d later. It should be expected that, even if these cows failed to conceive to the timed insemination, most cows would be synchronized after the Ovsynch/TAI protocol. Thus, assuming that the normal length of estrous cycle varies between 17 to 24 days, cows initiated the second-service Ovsynch/TAI protocol between days 8 to 15 of the estrous cycle. Therefore, it can be hypothesized that most cows were not in the optimal stages of the estrous cycle at initiation of the second-service Ovsynch/TAI and, as a consequence, had reduced pregnancy rates. Such a problem may be minimized whether cows are re-synchronized at 29 d after first-service. In this manner, the second-service Ovsynch/TAI protocol would be initiated between 5 to 12 d of the estrous cycle.

There is still much variation in the return to estrus of cows that fail to conceive after the first-service Ovsynch/TAI protocol because of short interestrous intervals and late embryonic losses. Such variation may affect the frequency of cows in the optimal stages of the cycle at initiation of the second-service Ovsynch/TAI protocol. Because inseminated cows should not be re-synchronized with $PGF_{2\alpha}$ prior to the second-service Ovsynch/TAI, an alternative solution may be to synchronize

cows with progesterone. Figure 6.2 depicts such a strategy.

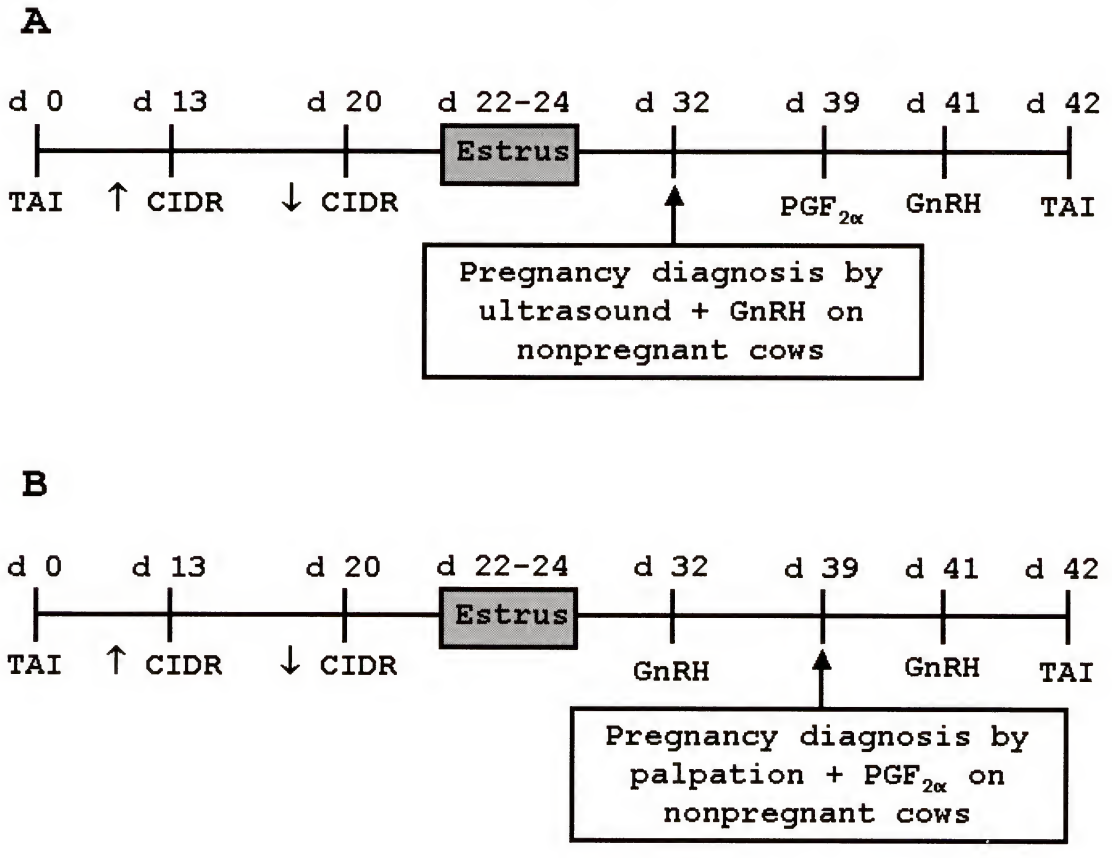

Figure 6.2. Schematic representation of a protocol that allows for pre-synchronization of a second-service Ovsynch/TAI protocol. Arrows indicate when CIDR devices would be inserted (↑) or removed (↓).

According to the second-service pre-synchronization method above, cows would receive progesterone supplementation (e.g. a CIDR device) on d 13 after first-service timed insemination. The CIDR device would be removed 7 d later (i.e., 20 d after first-service timed insemination) and most cows not conceiving to the first-

service should be expected to return to estrus within 2 to 4 days. The second-service Ovsynch/TAI would be initiated at 32 d after first-service timed insemination and most cows would be targeted to initiate the Ovsynch/TAI protocol between d 8 to 10 of the cycle. At this point, the producer/veterinarian has two options: all cows may be examined with an ultrasound at d 32 and only those diagnosed open would initiate the second-service Ovsynch/TAI protocol (Figure 6.2, Panel A). Alternatively, cows may be injected with GnRH at d 32 and then, 7 d later, cows would be palpated for pregnancy and only open cows would receive a $PGF_{2\alpha}$ injection, followed by a GnRH injection 2 d later, and cows would be timed inseminated the following day (i.e., 42 d after first-service timed insemination; Figure 6.2, Panel B). Although such a system would require an intensive management of cows, it provides producers with the opportunity to maximize pregnancy rates after re-synchronization and may completely eliminate the need for estrous detection.

The experiment in Chapter 3 also indicated that initiation of bST treatment at the first injection of GnRH of the Ovsynch/TAI protocol or at the time of insemination increased first-service pregnancy rates. This finding agrees with previous observations by Moreira et al.

(2000d). The effects of bST on pregnancy rates were exerted after insemination because no differences were observed between cows initiating bST treatment at first GnRH or at the time of insemination. Thus, the increase in pregnancy rates due to bST does not seem to be dependent upon timed insemination.

In Chapter 2 it was discussed that one of the potential effects of bST that causes decreased reproductive performance is lower estrous detection rates. The use of bST may directly affect behavioral centers within the brain to inhibit estrous expression (Kirby et al., 1997b). Such an effect would not be present if bST treatment were to be initiated only after estrus is detected. This way, bST would not interfere with estrous detection and would be able to express its beneficial effects on conception rates. Hernandez-Ceron et al. (2000) injected bST at the time of insemination and observed an increase in pregnancy rates of cows having more than one service. Bilby et al. (1999) treated dairy and beef cows with bST at insemination and also observed a trend for increased pregnancy rates, although it was not statistically significant. However, it must be pointed out that researchers used a third of the dose (i.e., 167 mg) used by Moreira et al. (2000d) and Hernandez-Ceron et al. (2000) in the latter experiment.

Collectively, this indicates that bST increases conception rates because of its effects after insemination.

The experiments described in Chapter 4 and 5 were designed to provide further evidence that bST may increase pregnancy rates and to provide further insight as to the mechanisms by which bST exerts its effects. The experiment in Chapter 4 used an in vivo model where cows were superovulated to increase the chances to observe a bST effect on early embryonic development. A second part of this same experiment was designed to observe whether such embryos would have a greater survival rate after transfer and whether treatment of recipient cows with bST also would affect subsequent pregnancy rates. In Chapter 5, the experiment used an in vitro model to determine whether GH or IGF-I directly and independently affected oocyte maturation and early embryonic development.

The experiment in Chapter 4 indicated that a single dose of bST injected at insemination of superovulated donor cows decreased the number of unfertilized ova recovered at embryo flushing 7 d after insemination. Such an effect, coupled with the lack of differences in the total number of structures flushed at d 7, was interpreted to indicate that bST stimulated oocyte maturation and increased fertilization rates after insemination. Further evidence

of such a stimulatory effect of bST on fertilization rates was based on the observation that the percentage of embryos classified as transferable also was increased by bST treatment. In a series of experiments described in Chapter 2, Izadyar et al. (1996, 1997a, 1997b, 1998a, 1998b) described that addition of GH to the maturation medium of bovine oocytes improved maturation and subsequent fertilization and cleavage rates. These observations were supported by results in the in vitro experiment described in Chapter 5, where addition of GH during maturation stimulated subsequent cleavage rates. Collectively, these observations indicate that bST may be increasing conception rates by enhancing maturation of the oocyte and increasing its capacity to undergo fertilization upon insemination.

Data from Chapter 4 also indicated that early embryonic development was enhanced when superovulated cows received bST treatment. The number of blastocysts at embryo flushing was increased and more embryos were in advanced stages of development among bST-treated donors. Similar results also were observed in the in vitro study described in Chapter 5. Addition of GH or IGF-I during embryo culture increased the percentage of blastocysts and also stimulated embryo development to more advanced stages. Evidence for a direct effect of GH was provided by the

observation addition of anti-IGF-I did not eliminate the stimulatory effect of GH. Effects of both GH and IGF-I to stimulate embryonic development during in vitro culture of bovine embryos were detected (Izadyar et al., 2000; Kaye et al., 1992; Matsui et al., 1994; Palma et al., 1997). Evidence that both GH and IGF-I may play a physiological role during early embryonic development was presented in Chapter 2 and is summarized in a model depicted in figure 6.3.

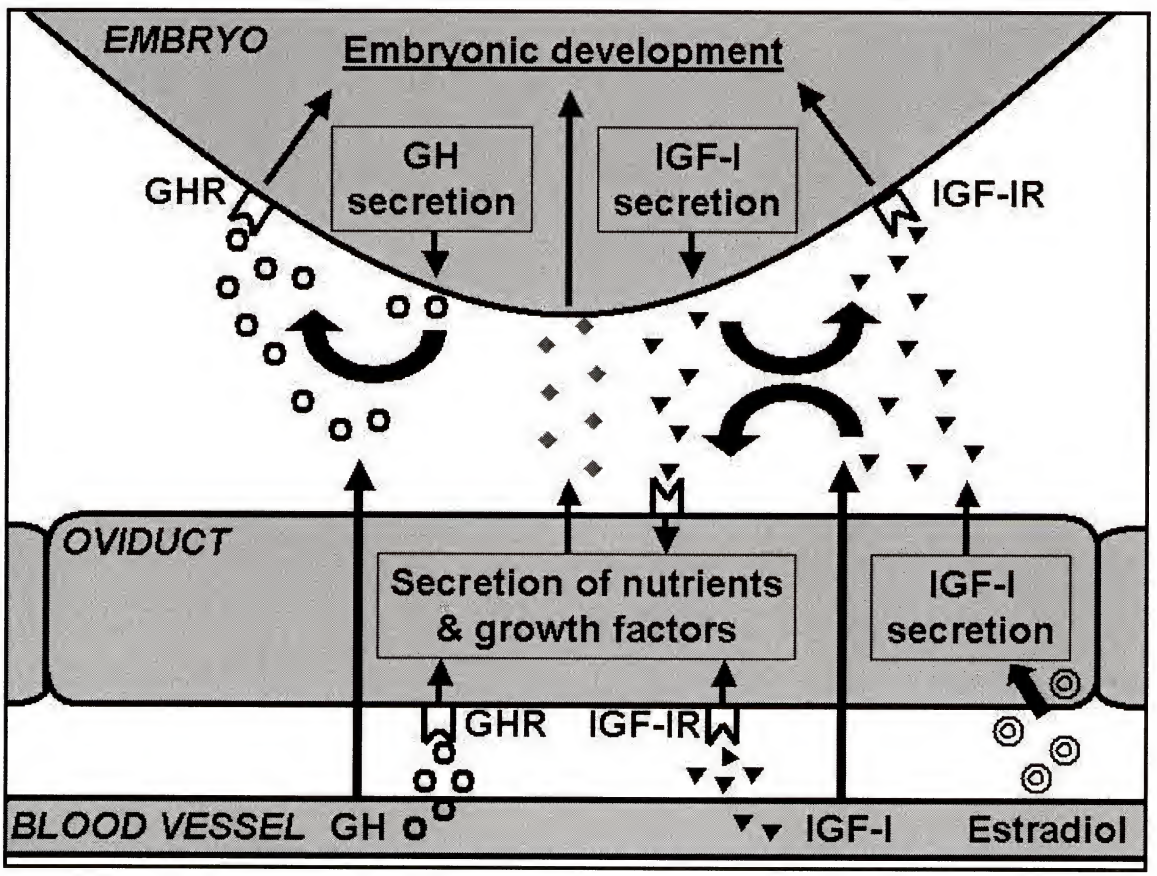

Figure 6.3. Schematic model for the effects of GH and IGF-I during early embryonic development.

According to the present model, both GH and IGF-I circulating in plasma may bind to their respective receptors on the oviduct (Kirby et al., 1996; Lucy et al., 1995a; Schmidt et al., 1994; Stevenson and Wathes, 1996) and potentially stimulate the secretion of nutrients and other growth factors into the lumen that may stimulate embryonic development. Also, it was demonstrated that, during the periovulatory period, concentrations of IGF-I are increased within the oviduct and uterus of cows (Schmidt et al., 1994), and such an effect is probably mediated by estradiol (Wathes et al., 1998). Other sources of IGF-I include the developing embryo (Lonergan et al., 1999; Lonergan et al., 2000; Watson et al., 1992; Watson et al., 1994) and, possibly, plasma IGF-I may reach the oviductal lumen. IGF-I within the oviductal lumen may bind to receptors in the embryo (Watson et al., 1992) and stimulate embryonic development (Herrler et al., 1992; Kaye et al., 1992; Matsui et al., 1994) or bind to the oviduct epithelium to stimulate the secretion of nutrients and other growth factors. The embryo also is able to produce GH (Lechniak et al., 1999), which may act in an autocrine fashion and bind to GH receptors on the embryo to stimulate development (Izadyar et al., 2000). In addition, it may be

possible that GH from plasma may reach the oviductal lumen and stimulate embryonic development.

After the embryo moves from the oviduct and reaches the lumen of the uterine horn, there is further evidence that the IGF-I system may play a role on its development. Concentrations of IGF-I within the uterine lumen are decreased during the luteal phase of the cycle (Geisert et al., 1991), but expression of IGF-I receptors was highest in the mid-luteal phase (Wathes et al., 1998) and IGF-I receptors were localized mainly in the epithelium of the endometrial glands (Stevenson et al., 1994; Wathes et al., 1998). The presence of IGF-I receptors on uterine epithelial glands suggests a role for IGF-I in regulating their secretory activity that is likely to be important in providing an environment capable of sustaining embryonic. Existence of a role for GH and/or IGF-I during embryonic development after d 7 was supported by the observation that recipient cows that received bST treatment at estrus had increased pregnancy rates after embryo transfer, as demonstrated in Chapter 4.

Collectively, these observations support the hypothesis that administration of bST does increase pregnancy rates of lactating dairy cows when administered at insemination. Such an effect appears to be involved

with oocyte maturation and early embryonic development and may involve both embryonic and maternal factors. Present evidence does not support a role for GH and/or IGF-I to stimulate embryonic development and survival after the period of maternal recognition of pregnancy because embryonic losses after d 18 of pregnancy (Moreira et al., 2000b) or after d 32 of pregnancy (Chapter 3) were not affected by bST treatment. However, many questions remain to be answered.

As described, evidence indicates that GH improves maturation of the oocyte and stimulates fertilization rates. Moreover, both GH and IGF-I seem to stimulate early embryonic development. An important question is whether such effects of both GH and IGF-I can be intensified after an injection of bST. It is well accepted that an injection of bST will increase plasma concentrations of both GH and IGF-I. Data discussed in Chapter 2 also indicates that follicular fluid concentrations of both GH and IGF-I are increased after administration of bST, which supports the hypothesis that treating cows with bST may stimulate oocyte maturation. Not much is known, however, about whether concentrations of GH and IGF-I within oviductal and uterine lumen are increased after bST treatment. One experiment examined the effect of bST treatment on uterine flushings

during the late diestrus (i.e., 17 d after insemination; Lucy et al., 1995b). The concentration of IGF-binding proteins 3, 4, and 5 were increased in the uterine luminal fluid after cows received bST treatment, but IGF-I concentrations were unaltered (Lucy et al., 1995b). It may be possible that the effects of bST on embryonic development may be related to the expression of IGF-binding proteins that regulate the availability of IGF-I.

Nonetheless, the effects of a sustained-release full dose of bST on IGF-I and GH concentrations within the oviduct and uterine lumen during the period of early embryonic development have not been examined. This is one missing link in the present hypothesis that needs to be further investigated. Because expression of IGF-I by the oviductal and uterine epithelium is increased during the periovulatory period (Schmidt et al., 1994; Geisert et al., 1991), an injection of bST at this time may produce an additive effect and increase the secretion of IGF-I and/or alter the expression of IGF-binding proteins.

Increased GH and IGF-I concentrations within the lumen of the oviduct and uterus may be due to an effect of GH and/or IGF-I on oviductal/uterine epithelial cells to stimulate the secretion of IGF-I. There is no evidence at this point that the oviductal or uterine epithelium is able

to secrete GH. Alternatively, plasma GH and IGF-I can reach both oviductal and uterine epithelium an act directly on the early embryo. Subsequent research must attempt to provide answers for these questions and examine more thoroughly regulation of the GH and IGF-I system (IGF-I, IGF-II, IGF-binding proteins, and IGF receptors).

Results from the present series of experiments provide no evidence that use of bST has any detrimental effects on pregnancy rates to first or subsequent services. Instead, producers may be able to use bST as recommended by the manufacturer to enhance first service pregnancy rates. Such an observation may have a significant impact on the reproductive efficiency of dairy herds because bST is used widely within the United States.

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BIOGRAPHICAL SKETCH

Frederico Augusto Pena Moreira was born on June 4, 1970, to Auricedes Alves Moreira and Tereza Lúcia Pena in Curvelo, Brazil. He is the older of two children. He was accepted at the Federal University of Minas Gerais in 1988 where he received the degree of Médico Veterinário (Brazilian equivalent of D.V.M.) in July 1993. After his graduation, he participated on an exchange program at a dairy operation in Georgia, USA, where he remained until December 1994. For the next 6 months, he practiced animal medicine in Curvelo, Brazil. In August 1995 the author enrolled in the graduate program of the Department of Animal Sciences at the University of Florida where he completed his master's research under the supervision of Dr. William W. Thatcher (Graduate Research Professor) in December 1998. The author remained at the University of Florida to pursue a doctoral degree. After completion of the requirements for a Doctor of Philosophy degree, he will work for a pharmaceutical industry.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William W. Thatcher, Chair
Graduate Research Professor, Animal Sciences

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Peter J. Hansen
Professor of Animal Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Lokenga Badinga
Assistant Professor of Animal Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Joel Yelich
Assistant Professor of Animal Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jorge Hernandez
Assistant Professor of Veterinary Medicine

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 2001

E. Jane Ingram

Dean, College of Agricultural and Life Sciences

Dean, Graduate School